# Characterization and Toxicological Studies of Pigment from *Castanea mollissima*

**LEUNG Bo-Shan**

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**Thesis committee:**

Prof. Y.S. Wong (Supervisor)

Prof. P.K. Wong

Prof. Vincent E.C. Ooi

Prof. Patrick Leung (External Examiner)

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# Abstract

Colorants are added to food items to give consumers the appetizing and attractive qualities they desire. Caramel, a brown food colorant, has been widely used in food industries. Studies indicated that ammonia (Class III) caramel may have undesirable effects on the immune system of the experimental animals. The consumers' concern on the safety of synthetic food additives remains and the demand for natural pigments as food colorants increases.

In this research, a natural brown pigment was prepared from the Chinese chestnut (*Castanea mollissima*) shells. This *Castanea* pigment (CP) preparation was shown to be stable at autoclaving temperature (121°C). Its color remained unchanged after a 12-month test period under normal room light condition. The CP was also found to possess antioxidative activity.

The Microtox® test indicated that the toxicity of the crude CP was very low (EC50 = 360 ppm). The toxicity was further reduced by subsequent purification procedures including organic solvent extraction (EC50 = 650 ppm). The mutagenicity of CP was assessed by the Mutatox® test. Results showed that CP was not a suspected mutagen.

For the safety evaluation of CP, its toxicity was further assessed using the experimental animal model. CP dissolved in distilled water was provided for drinking *ad libitum* to male Sprague-Dawley (SD) rats. An acute toxicity test was run for 14 days with 0 and 8% CP, while the chronic tests were run for 6 months and 9 months with 0, 0.25 and 1% CP. Physical parameters including the body weight gains, food and fluid consumptions were recorded during the course of the experiment. Clinical analysis including haematology, blood chemistry and

urinalysis were done. Histopathological examination was performed for organs including kidney, liver and thymus, also testis for the chronic test. All rats tolerated these doses of CP well. No adverse effect was found in the acute test except an increase in the caecal content and reductions in serum aspartate aminotransferase (AST) and γ-glutamyl transpeptidase (GGTP) levels were observed in treated rats. In the chronic test, no rats treated with CP showed any clinical signs of toxicity.

This study suggested that CP is a safe natural food colorant.

# 摘要

利用色素對食品進行着色能吸引消費者及增加他們的食慾。焦糖是一種棕色食用色素，廣泛應用於食品工業。有研究顯示第三類氨焦糖會對實驗動物的免疫系統產生不良影響。消費者對於人工合成色素的安全性十分關注，天然色素作為食用色素的需求亦因而大大增加。

在這項研究中，一種天然棕色素從板栗殼中提取出來。研究結果顯示板栗色素在高壓反應器下(121°C)保持穩定。經過十二個月的室溫光線存放下，它的顏色仍維持不變。此外，板栗色素具有抗氧化活性。

在 Microtox®測試中，粗製的色素毒性很低(EC50 = 360 ppm)，而有機溶劑提純過程能使毒性進一步下降(EC50 = 650 ppm)。除此之外，Mutatox®測試顯示板栗色素不是一種誘變的物質。

在評鑑板栗色素的安全性中，其毒性測試再進一步利用實驗動物來進行。板栗色素溶解於蒸餾水提供給雄性 Sprague-Dawley(SD)大鼠不斷食用。一項急性毒性測試進行為期十四天，採用 0% 和 8% 濃度的板栗色素；而慢性毒性測試則進行為期六個月和九個月，採用 0%、0.25% 和 1% 濃度的板栗色素。基本因素包括體重增加、食糧和飲水的消耗量在實驗過程中記錄下來。進行的臨床分析包括血液

學、血液化學和尿液測試。組織病理學的檢查則包括腎臟、肝臟、胸腺和睪丸。結果顯示，所有大鼠均能容忍這些劑量。在急性毒性測試中，除了發現盲腸的含量偏高及血清中天門冬氨酸轉氨酶(AST)和丙麩氨酸轉氨酶(GGTP)的水平偏低外，並沒有其他不利的效果產生。而在慢性的測試中，均沒有任何跡象顯示板栗色素帶有毒性。

這項研究結果顯示板栗色素可以成為一種天然食品色素。

# Acknowledgements

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# List of Abbreviations

| | |
|---|---|
| AC | Ammonia caramel |
| ADI | Acceptable daily intake |
| ADP | Adenosine-5-diphosphate |
| A/G ratio | Albumin/Globulin ratio |
| ALP | Alkaline phosphatase |
| ALT | Alanine aminotransferase |
| ANOVA | Analysis of variance |
| AST | Aspartate aminotransferase |
| ATP | Adenosine-5-triphosphate |
| BHA | Butylated hydroxyanisole |
| BHT | Butylated hydroxytoluene |
| CP | *Castanea* pigment |
| DAP | Dihydroxyacetone phosphate |
| $DPPH^{\bullet}$ | 1,1-diphenyl-2-picryhydrazyl radical |
| EC50 | Effective concentration exhibiting 50% inhibition |
| EEC | European Economic Community |
| ELISA | Enzyme–linked immunosorbent assay |
| FDA | Food and Drug Administration |
| GGTP | γ-glutamyl transpeptidase |
| GK | Glycerol kinase |
| G-1-P | Glycerol-1-phosphate |
| G-1-PDH | Glycerol-1-phosphate dehydrogenase |
| Hb | Haemoglobin |
| HDL | High density lipoprotein |
| H&E | Hematoxylin and eosin |
| HPLC | High performance liquid chromatography |
| INT | 2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyltetrazolium chloride |
| ITCA | International Technical Caramel Association |
| $LD_{50}$ | Concentration exhibiting 50% mortality |

| | |
|---|---|
| LDL | Low density lipoprotein |
| MCH | Mean cell haemoglobin |
| MCHC | Mean cell haemoglobin concentration |
| MCV | Mean cell volume |
| NAD | Nicotinamide adenine dinucleotide |
| NADH | Nicotinamide adenine dinucleotide (reduced form) |
| PBS | Phosphate buffered saline |
| PBS-T | Washing buffer |
| PCV | Packed cell volume |
| RBC | Red blood cells |
| ROS | Reactive oxygen species |
| SD | Standard deviation |
| THI | 2-acetyl-4(5)-tetrahydroxybutylimidazole |
| TMB | 3,3',5,5'-tetramethylbenzidine |
| VLDL | Very low density lipoprotein |

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# Chapter 1 Introduction

## 1.1 Food colorants

Colorants have been used to make food more attractive and appetizing for centuries. It is often the first sensory quality by which foods are judged. Similarly, both food quality and flavor are associated with color. Our senses are trained to expect foods of certain colors and reject any deviations from our expectations. Foods that are aesthetically pleasing are more likely to be consumed, thereby contributing to a variety of diet and, hence, better nutrition.

Both synthetic and natural food colorants are used. They play a significant role in enhancing the aesthetic appeal of processed foods. Also, they are important ingredients in many convenience foods such as confectionery, instant desserts, ice-creams, snacks, and beverages, since many of these are naturally colorless. Colors are used to supplement the natural appearance of a given food system and to ensure batch-to-batch uniformity where raw materials have varying color intensities.

However, more and more synthetic food colorants have been proven to be toxic. Table 1.1 lists the synthetic colorants which were banned in the United States since 1950s (Ghorpade *et al.*, 1994). For example, Amaranth, which was first used in 1907, was banned in 1976. It has been misused for about seventy years. This provides a message that synthetic food colorants which are considered safe now, may not be safe any more in the future. There are now only seven dyes that have been approved by the Food and Drug Administration (FDA) for safe use in food, drugs and cosmetics. These are known as FD&C dyes. These dyes are Yellows #5 and #6, Reds #3 and #40, Blues #1 and #2, and Green #3. The most popular of these dyes is Red #40. The second most popular is Yellow #5 (Ghorpade *et al.*, 1994).

**Table 1.1 Synthetic food colorants banned in the United States.**

| *Common name* | *FDA name* | *Year listed for food use* | *Year delisted* | *Current status for food use* |
|---|---|---|---|---|
| Ponceau 3R | FD&C Red No.1 | 1907 | 1961 | Not allowed |
| Amaranth | FD&C Red No.2 | 1907 | 1976 | Not allowed |
| Orange 1 | FD&C Orange No.1 | 1907 | 1956 | Not allowed |
| Naphthol Yellow S | FD&C Yellow No.1 | 1907 | 1959 | Not allowed |
| Light Green SD Yellowish | FD&C Green No.2 | 1907 | 1966 | Not allowed |
| Yellow AB | FD&C Yellow No.3 | 1918 | 1959 | Not allowed |
| Yellow OB | FD&C Yellow No.4 | 1918 | 1959 | Not allowed |
| Guinea Green B | FD&C Green No.1 | 1922 | 1966 | Not allowed |
| Ponceau SX | FD&C Red No.4 | 1929 | 1976 | Not allowed |
| Orange SS | FD&C Orange No.2 | 1939 | 1956 | Not allowed |
| Oil Red XO | FD&C Red No.32 | 1939 | 1956 | Not allowed |
| Benzyl Violet 4B | FD&C Violet No.1 | 1950 | 1973 | Not allowed |

(Ghorpade *et al.*, 1994)

## 1.2 Caramel

Caramel is probably the most widely used food colorants in foods & drinks. It belongs to the group of melanoidin pigments (Rayner, 1991). This group of compounds is responsible for the attractive red-brown color of cooked foods. When carbohydrates are heated at high temperatures, they caramelize to produce a characteristic flavor and color. Melanoidin pigments are responsible for the color of caramels, malt extracts, and toasted carob flour (Rayner, 1991). The FDA defines caramel as " the dark brown liquid or solid material resulting from the carefully controlled heat treatment of food-grade carbohydrates such as glucose, dextrose, lactose and sucrose" (Marmion, 1984).

The European Economic Community (EEC) has assigned an E number, i.e E150, for caramel in 1962. In 1985, the International Technical Caramel Association (ITCA) classified it into 4 distinct classes according to manufacture and usage. In addition to its functions as food colorants, caramel has other valuable functional properties. It slowly absorbs oxygen and stabilizes colloidal systems, thus facilitating flavor retention as well as preventing hazes in products such as beers. Caramel also has emulsifying properties to facilitate the dispersion of water-insoluble materials, such as flavoring oils, in an aqueous phase (Chappel and Howell, 1992).

### 1.2.1 Classes of caramel

***Caramel I***

Class I caramel, E150a, is also called plain caramel or caustic caramel. Making class I caramel is the simplest among the 4 classes, it is prepared by the controlled heat treatment of carbohydrates with alkali or acid (Licht *et al.*, 1992).

The ITCA committee allocated to this class an acceptable daily intake (ADI) 'not specified' (Chappel and Howell, 1992). The major uses of this class include cookie fillings and pet food.

***Caramel II***

Class II caramel, E150b, is also called caustic sulphite caramel. Unlike class I, sulphite-containing compounds are added as catalysts in the heat treatment of carbohydrates (Licht *et al.*, 1992). No ADI was set (Chappel and Howell, 1992). Its usage is not common.

***Caramel III***

Class III caramel, E150c, is also called ammonia caramel (AC). Ammonia compounds are added in the heat treatment of carbohydrates (Licht *et al.*, 1992). An ADI of 0-200 mg/kg body weight was allocated to this class (Chappel and Howell, 1992). It is used extensively as a color additive in beers, sauces, gravies and baking products (Houben *et al.*, 1992).

***Caramel IV***

Due to the addition of ammonia-containing and sulphite-containing compounds (Licht *et al.*, 1992), class IV caramel (E150d) is also called ammonia-sulphite caramel. Similar to class III caramel, an ADI of 0-200 mg/kg body weight was established (Chappel and Howell, 1992). Its major uses are in soft drinks like Coca-Cola.

### 1.2.2 Toxicological studies of caramel

The 4 classes of caramel have been examined extensively by chemical analysis and safety testing. Class I, II and IV showed no toxicologically significant effects in mammals (Adams *et al.*, 1992; Chappel and Howell, 1992; MacKenzie *et al.*, 1992). However, the safety of caramel III has been questioned in recent studies (Sinkeldam *et al.*, 1988; Houben *et al.*, 1992; MacKenzie *et al.*, 1992). It has been shown that the presence of 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI), an agent widely used as a component of ammonia caramel food coloring, leads to several drawbacks on the immune system of rats (Houben *et al.*, 1992).

***Effects of class III caramel on the immune system of rats***

Administration of class III caramel has been associated with a reduction in total white blood cell and lymphocytes counts in rats (Sinkeldam *et al.*, 1988; Houben *et al.*, 1992). This effect is caused by the imidazole derivative 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI), an agent widely used as a component of ammonia caramel food coloring (Sinkeldam *et al.*, 1988; Houben *et al.*, 1992; MacKenzie *et al.*, 1992).

The reduction of total white blood cell counts occurred in spleen and popliteal lymph node, as well as in blood. Also, a stronger decrease in CD4+ (T-helper) cells than in CD8+ (T-cytotoxic) cells was observed (Houben *et al.*, 1992).

Hematoxylin and eosin staining of serial thymic sections of AC administered rats showed a decrease in cortical area and an enlargement of the medulla (Figure 1.1) (Houben *et al.*, 1992). Moreover, an increased cell density was observed in the medulla.

With the use of immunohistochemical staining techniques, the increase in medullary cell density was characterized as an increase in the number of mature

medullary thymocytes, mainly of the CD4+ phenotype. This observation, together with a decrease in the number of ER4+ cells, the recent thymus emigrants observed in spleen. This indicated a diminished migration of mature thymocytes from the thymus into the periphery. The changed lymphocyte migration patterns were suggested to play a role in the lymphopenia caused by THI (Houben *et al.*, 1992).

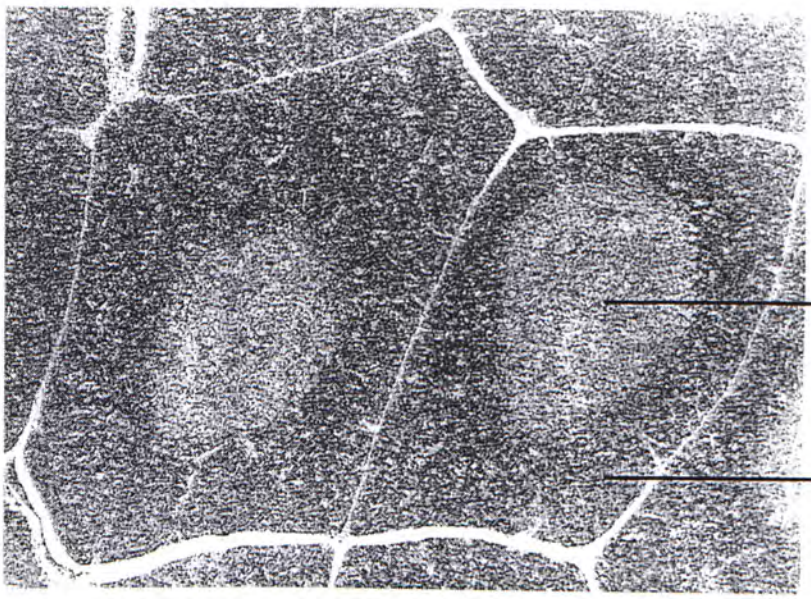
Further research indicated that most of these effects occurred only when the animals were fed with a diet low in vitamin B6. Addition of vitamin B6 to the diets of Caramel Color III-exposed rats can prevent the reduction in the number of blood lymphocytes (Noltes and Chappel, 1985; Conway and Paine, 1988; Sinkeldam *et al.*, 1988).

A summary of the most important effects of caramel III and THI on the rat immune system is presented in Table 1.2.

**Table 1.2 Summary of the most important effects of caramel III and THI on the rat immune system.**

| Parameter | Observation |
|---|---|
| ***Body and organ weights*** | |
| Body weight | No effect |
| Thymus weight | No effect |
| Spleen weight | Decreased |
| ***Cell counts*** | |
| White blood cell counts | Decreased |
| Blood lymphocyte counts | Decreased |
| Cell number bone marrow | No effect |
| Cell number thymus | No obvious effect |
| Cell number spleen | Decreased |
| Cell number lymph nodes | Decreased |
| ***Lymphocyte subsets*** | |
| Bone marrow | No effect |
| Blood, spleen, popliteal lymph nodes | B/T ratios not affected, CD4+/CD8+ ratios decreased |
| ***Histology*** | |
| Thymus | Cortex/medulla area ratios decreased, increased cell density in medulla |
| Spleen | Lymphocyte depletion, increased numbers of megakaryocytes in red pulp |
| Lymph nodes | Lymphocyte depletion |
| ***Immuno-histochemistry*** | |
| Thymus | Increased numbers of mature medullary cells, reduced numbers of ED2+ macrophages in cortex |
| Spleen | Reduced number of ER4+ cells (recent thymus emigrants), reduced numbers of ED2+ macrophages in red pulp |
| ***Immune functions*** | |
| T-cell dependent antibody responses | Impaired |
| Cell-mediated immune responses | Impaired |
| Natural Killer cell activity of spleen cells | Decreased |
| Natural Killer cell activity of peritoneal cells | No effect |
| Mitogen-induced proliferation of spleen cells | Decreased |
| Mitogen-induced proliferation of thymocytes | Increased |

(Houben and Penninks, 1994)

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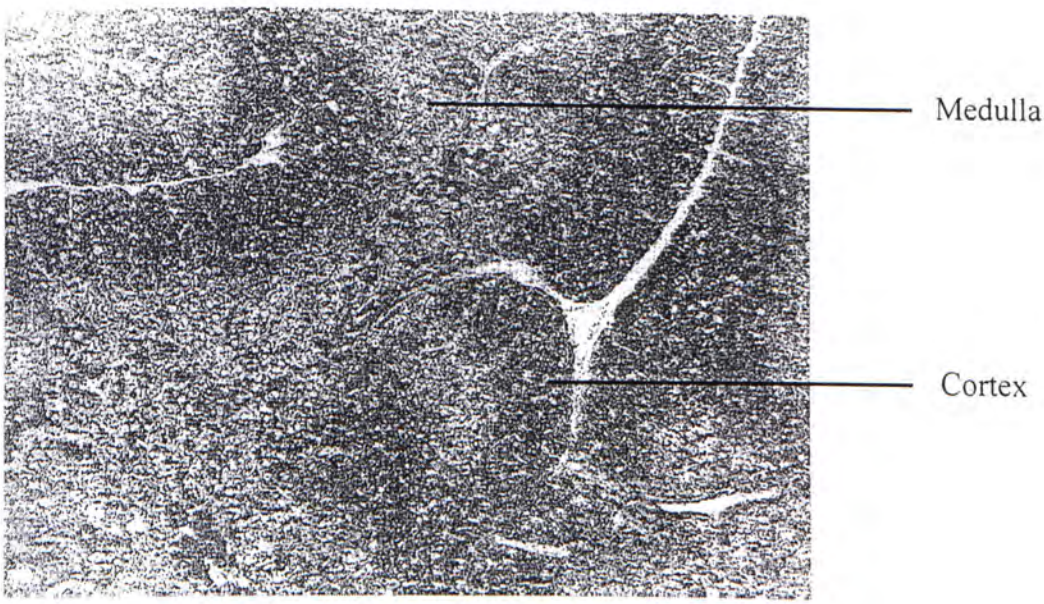


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**Figure 1.1 Photomicrographs of thymus sections of rats.**
(A) a control rat; (B) a THI-exposed rat. (Houben *et al.*, 1992).

## 1.3 *Castanea mollissima*

*Castanea mollissima,* family Fagaceae, is commonly called Chinese chestnut. It reaches a height of 40 to 60 inches in a sunny exposure and a well drained soil. Leaves are simple, alternate, and deciduous with bristle-tipped and a hairy underside. They are 5 to 8 inches long. Flowers are present in June. Male flowers are small and white, 4 to 5 inches long. Female flowers are 1/2 to 3/4 inch long. Fruit a nut, which is edible and matures in September, 1 to 1 1/4 inches in diameter, brown, mostly round, but flattened on 1 to 2 sides, 2 to 3 in each bur.

## 1.4 Antioxidants

### 1.4.1 Background

***Free radicals and reactive oxygen species (ROS)***

Oxidants or reactive oxygen species (ROS) is a collective term that includes not only oxygen-derived radicals such as superoxide ($O_2^{\bullet-}$) but also some non-radical derivatives of oxygen such as hydrogen peroxide ($H_2O_2$) (Halliwell *et al.*, 1992). A free radical has one or more unpaired electrons and is unstable and more reactive than non-radical species. Some of the free radicals and other important oxidants found in living organisms are shown in Table 1.3 (Langseth, 1995).

If free radicals are not inactivated, their chemical reactivity can damage all types of cellular macromolecules such as proteins, carbohydrates, lipids, and nucleic acids. Some of the types of damage that can result from the actions of free radicals are shown in Figure 1.2 (Langseth, 1995). The free-radical reaction has been associated with the pathology of several human diseases such as atherosclerosis and cancer (Anderson and Phillips, 1999).

***Lipid peroxidation***

Lipid peroxidation contributes to the development of cardiovascular diseases such as atherosclerosis (Brown and Goldstein, 1990). Also, the end products of this process can bring damage to protein and DNA. The oxidative modification of low-density lipoprotein (LDL) may be a key early step in atherogenesis. Macrophages, having the ability to oxidize LDL, take up the oxidized LDL rapidly and without regulation via scavenger receptors. Hence, it leads to the accumulation of cholesterol and the formation of foam cells (Fuller and Jialal, 1997). These foam cells contain large cytoplasmic deposits of cholesteryl ester and free cholesterol in

lipid droplets, are thought to be the earliest indicator of cardiovascular disease (Goldstein *et al.*, 1979; Parthasarathy, 1994; Stone *et al.*, 1996; Dragsted, 1998).

The free-radical reaction of lipid peroxidation is important in the food industry and it may affect the food safety and appearance (Dragsted, 1998).

***Oxidative stress***

The human body has several mechanisms for defense against free radicals and reactive oxygen species. The various defences are complementary to one another because they act on different oxidants or in different cellular compartments. The examples of endogenous antioxidants are glutathione peroxidase (Langseth, 1995), superoxide dismutase (Halliwell, 1996) and catalase (Elstner *et al.*, 1994). These enzymes can decrease the concentration of the most harmful oxidants.

However, if exposure to exogenous sources of oxidants is high, the body's antioxidant defenses may be unable to cope. The imbalance between pro-oxidants and antioxidants is called oxidative stress (Gutteridge and Halliwell, 1994).

***Role of antioxidants***

An antioxidant is any substance that delays or inhibits, oxidative damage to a target molecule such as lipid, protein, nucleic acid and carbohydrate (Gutteridge and Halliwell, 1994).

Exogenous antioxidants are available in fruits, vegetables and beverages. The synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), act as food additives can prevent peroxidation and improve the stability of lipids and lipid-containing foods (Onyeneho and Hettiarachy, 1991). Their chemical structures are shown in Figure 1.3. These antioxidants can terminate the free radical chains in the lipid oxidation by acting as electron donors.

Natural antioxidants such as vitamin C (ascorbic acid) and carotenoids are found in fruits and vegetables. Ascorbic acid, acts as an oxygen scavenger, can react with oxygen and remove it in a closed system. A high consumption of food containing these antioxidants has been proved to be effective in preventing cancer and heart disease (Pryor, 1993; Steinmetz and Potter, 1996).

Moreover, phenolic compounds are widely distributed in plants. One of the major groups of phenolic compounds is the flavonoids, which are important in contributing to the flavor and color of many fruits and vegetables and products derived from them such as wine, tea and chocolate. This is now much interest in the biological effects of phenolic compounds since evidence was found that diets rich in fruit and vegetables appear to protect against cardiovascular disease and some forms of cancer (Bors *et al.*, 1990; Rice-Evans *et al.*, 1996; Cook and Saman, 1996). Some dietary sources of flavonoids and phenolic acids are shown in Table 1.4 (Cook and Saman, 1996).

Nowadays, there is an increasing demand on the antioxidants from natural products. They are regarded as more safe and more appreciated than the synthetic antioxidants.

### 1.4.2 Methods used to evaluate the antioxidative activity

Lipid peroxidation is one of the most important processes of the food deterioration because it may affect food safety, color, flavor and texture. Antioxidants may protect food quality by preventing oxidative deterioration of lipids (Kinsella *et al.*,1993).

Several methods to determine free radical scavenging have been reported, the reaction with 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) have received more attention.

### 1.4.2.1 DPPH$^{\bullet}$ scavenging method

DPPH$^{\bullet}$ scavenging method is based on the measurement of free radical scavenging of the antioxidant compounds using a stable radical, DPPH$^{\bullet}$ (Brand-Williams *et al.*, 1995) and it was widely used in the determination of the free radical scavenging ability of the food sources.

When DPPH$^{\bullet}$ is mixed with methanol, it shows a strong absorption band at 515 nm and appears in violet color. However, after the addition of antioxidant (AH), DPPH$^{\bullet}$ is reduced and the absorbance decreases. Hence, in order to determine and compare the free radical scavenging ability of the antioxidants in food sources, the decreasing rate in the absorbance gives much information.

$$DPPH^{\bullet} + AH \longrightarrow DPPH\text{-}H + A^{\bullet}$$

### 1.4.2.2 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a powerful chromatographic technique. It can often easily achieve separations and analyses that would be difficult or impossible using other forms of chromatography. HPLC is the term used to describe liquid chromatography in which the liquid mobile phase is mechanically pumped through a column that contains the stationary phase.

Reversed-phase (RP) chromatography is the most widely used chromatographic mode used in food items (Lea, 1988) and it is used to separate neutral molecules in solution on the basis of their hydrophobicity. RP-HPLC involves the use of a non-polar stationary phase and a polar mobile phase.

There are two types of elution methods. Isocratic elution is used when the sample is introduced onto the column and eluted from it under the same set of mobile phase conditions. Gradient elution using two or more solvents, is most commonly performed with RP-HPLC. It involves a continuous change in the composition of

the mobile phase to achieve separation of sample components of widely varying affinities for the stationary phase. In the separation, the most polar component elutes first, followed by the non-polar component (Lindsay, 1992).

In this study, RP-HPLC was used in the separation of CP. Fractions were collected to assess the antioxidative activity using $DPPH^{\bullet}$ scavenging method.

**Table 1.3 Some important reactive oxygen species in living organisms.**

| | |
|---|---|
| **Free radicals** | |
| Hydroxyl radical | $OH^{\bullet}$ |
| Superoxide radical | $O_2^{\bullet -}$ |
| Nitric oxide radical | $NO^{\bullet}$ |
| Lipid peroxyl radical | $LOO^{\bullet}$ |
| **Nonradicals** | |
| Hydrogen peroxide | $H_2O_2$ |
| Singlet oxygen | $^1O_2$ |
| Hypochlorous acid | HOCl |
| Ozone | $O_3$ |

(Langseth, 1995)

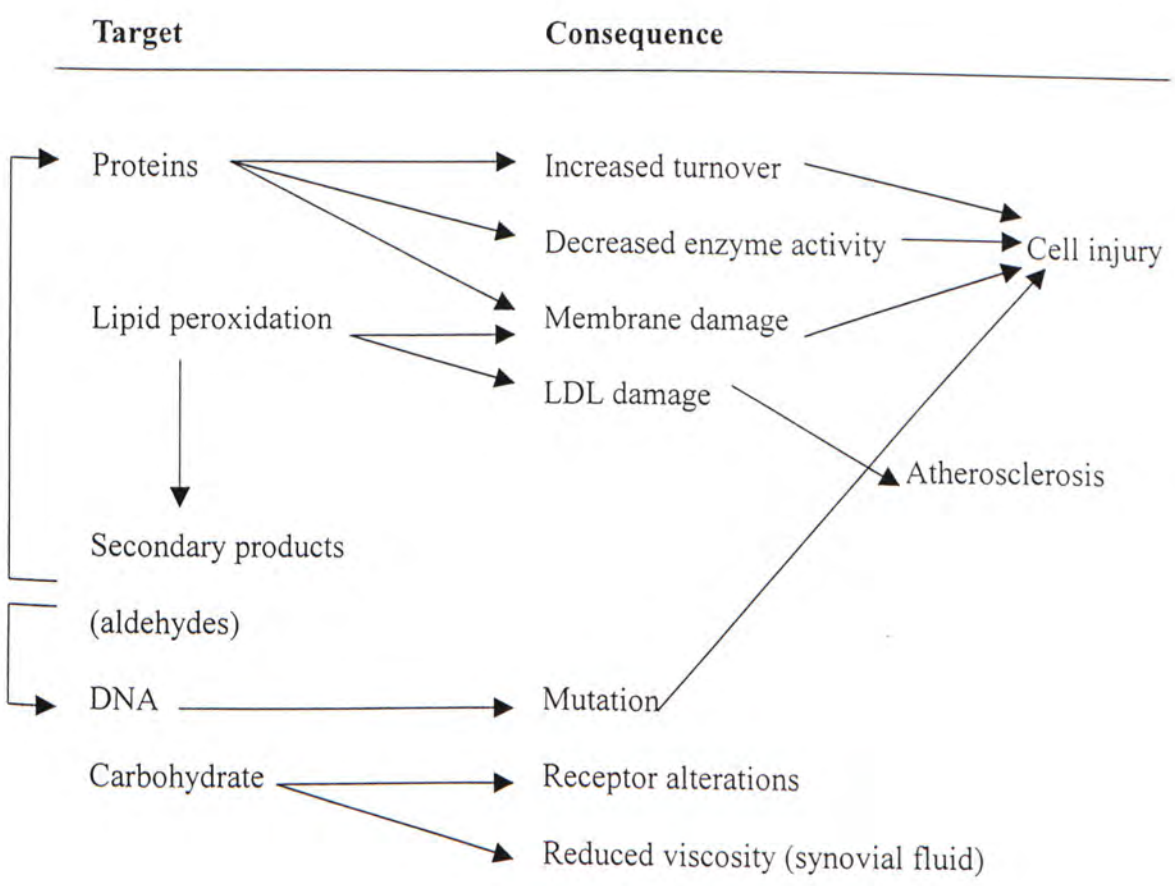
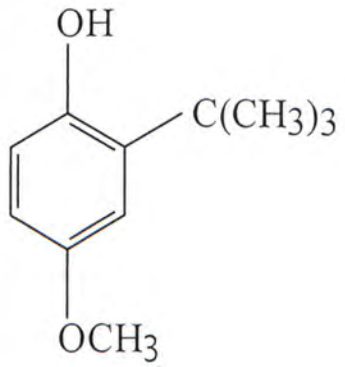
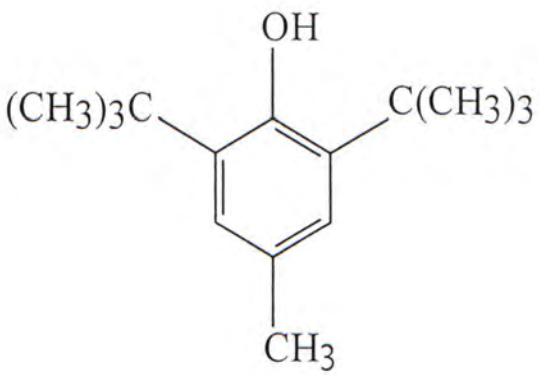



**Figure 1.2 Free radical damages.** (Langseth, 1995)




**Butylated hydroxyanisole (BHA)**




**Butylated hydroxytoluene (BHT)**

**Figure 1.3 Chemical structures of synthetic antioxidants, BHA and BHT.**

**Table 1.4 Some dietary sources of flavonoids and phenolic acids.**

| **Flavonoid** | **Source** |
|---|---|
| Catechins | Tea, red wine |
| Flavanones | Citrus fruits |
| Flavonols (e.g. Quercetin) | Onions, olives, tea, wine, apples |
| Anthocyanidins | Cherries, strawberries, grapes |
| Caffeic acid | Grapes, wine, olives, coffee, apples, tomatoes, plums, cherries |

(Cook and Saman, 1996)

## 1.5 Microtox® test

Microtox® is the first commercial toxicity test using a living luminescent marine bacterium, *Vibrio fischeri* (formerly known as *Photobacterium phosphoreum*, NRRL number B-11177) (Bulich, 1979). When properly grown, luminescent bacteria produce light as a by-product of their cellular respiration. Cell respiration is fundamental to cellular metabolism and all associated life processes. Bacterial bioluminescence is tied directly to cell respiration, and any inhibition of cellular activity (toxicity) results in a decreased rate of respiration and a corresponding decrease in the rate of luminescence (Tothill and Turner, 1996). The more toxic the sample, the greater the percent light loss from the test suspension of luminescent bacteria. Bacterial bioluminescence has proven to be a convenient measure of cellular metabolism and consequently, a reliable sensor for measuring the presence of toxic chemicals in aquatic samples. Strain B-11177 was originally chosen for the acute and chronic tests because it displayed a high sensitivity to a broad range of chemicals.

## 1.6 Mutatox® test

Mutatox® test is a biological test which uses a special dark mutant of luminescent bacterium (*V. fischeri*, strain M169) to detect the presence of mutagenic or genotoxic agents. Unlike normal luminescent bacteria, strain M169 is a dark variant and produces very low light during its growth cycle. However, this strain exhibits increased light production when grown in the presence of sub-lethal concentrations of mutagenic agents (Azur Environmental, 1995).

## 1.7 Methods used to evaluate the functions of major organs

### 1.7.1 Liver

Liver, like pancreas, develops embryologically as a glandular outgrowth of the primitive gut. The major functions of the liver are, for examples, detoxification of metabolic waste products, destruction of spent red cells, synthesis and secretion of bile, synthesis of the plasma proteins and plasma lipoproteins and metabolic functions (Fox, 1999). Due to the many functions of the liver, no single test can be used alone and 'batteries' of tests are therefore applied in the assessment of patients with known or suspected liver disease. Liver function tests (e.g. bilirubin, aminotransferases, alkaline phosphatase, γ-glutamyl transferase, etc.) are most common used for screening and monitoring the progress of patients with liver diseases (Corless and Middleton, 1983; Johnson, 1989).

*Alanine aminotransferase (ALT)*

ALT is an enzyme produced in hepatocytes, the major cell type in the liver. ALT is often inaccurately referred to as a liver function test, however, its level in the blood tells little about the function of the liver. The level of ALT in the blood is increased in conditions in which hepatocytes are damaged or die. As cells are damaged and ALT leaks out into the bloodstream. All types of hepatitis (viral, alcoholic, drug-induced, etc.) cause hepatocyte damage that can lead to elevations in the serum ALT activity (Walmsley and White, 1994). The ALT level is also increased in cases of liver cell death resulting from other causes, such as shock or drug toxicity. The level of ALT may correlate roughly with the degree of cell death or inflammation, however, this is not always the case. An accurate estimate of

inflammatory activity or the amount cell death can only be made by liver biopsy (Chopra and Griffin, 1985).

***Aspartate aminotransferase (AST)***

AST is an enzyme similar to ALT but less specific for liver disease as it is also produced in muscle and can be elevated in other conditions. AST is also inaccurately referred to as a liver function test by many physicians. In many cases of liver inflammation, the ALT and AST activities are elevated roughly in a 1:1 ratio. In some conditions, such as alcoholic hepatitis or shock liver, the elevation in the serum AST level may be higher than the elevation in the serum ALT level (Hubbard and Mechan, 1997).

***Alkaline phosphatase (ALP)***

Alkaline phosphatase is an enzyme, or more precisely a family of related enzymes, produced in the bile ducts, intestine, kidney, placenta and bone. An elevation in the level of serum alkaline phosphatase, especially in the setting of normal or only modestly elevated ALT and AST activities, suggests disease of the bile ducts. Serum alkaline phosphatase activity can be markedly elevated in bile duct obstruction or in bile duct diseases such as primary biliary cirrhosis. Alkaline phosphatase is also produced in bone and blood activity can also be increased in some bone disorders (Cella and Watson, 1989).

***γ-glutamyl transpeptidase (GGTP)***

An enzyme produced in the bile ducts that, like alkaline phosphatase, may be elevated in the serum of patients with bile duct diseases. Elevations in serum GGTP, especially along with elevations in alkaline phosphatase, suggest bile duct

disease (Cella and Watson, 1989). Measurement of GGTP is an extremely sensitive test, however, and it may be elevated in virtually any liver disease and even sometimes in normal individuals. GGTP is also induced by many drugs, including alcohol, and its serum activity may be increased in heavy drinkers even in the absence of liver damage or inflammation (Fox, 1999).

***Bilirubin***

Bilirubin is the major breakdown product that results from the destruction of old red blood cells. It is removed from the blood by the liver, chemically modified by a process called conjugation, secreted into the bile, passed in the intestine and to some extent reabsorbed from the intestine (Hubbard and Mechan, 1997). Bilirubin concentrations are elevated in the blood either by increased production, decreased uptake by the liver, decreased conjugation, decreased secretion from the liver or blockage of the bile ducts. In cases of increased production, decreased liver uptake or decreased conjugation, the unconjugated or so-called indirect bilirubin will be primarily elevated. In cases of decreased secretion from the liver or bile duct obstruction, the conjugated or so-called direct bilirubin will be primarily elevated (Tennant, 1999). Many different liver diseases, as well as conditions other than liver diseases (e.g. increased production by enhanced red blood cell destruction), can cause the serum bilirubin concentration to be elevated. Most adult acquired liver diseases cause impairment in bilirubin secretion from liver cells that cause the direct bilirubin to be elevated in the blood. In chronic acquired liver diseases, the serum bilirubin concentration is usually normal until a significant amount of liver damage has occurred and cirrhosis is present. In acute liver disease, the bilirubin is increased relatively to the severity of the acute process. In bile duct obstruction, or diseases of the bile ducts such as primary biliary cirrhosis, the alkaline phosphatase

and GGTP activities are often elevated along with the direct bilirubin concentration (Marshall, 2000).

### 1.7.2 Kidneys

Kidney is a bean-shaped organ which removes nitrogenous wastes such as urea and excess substances such as mineral salts out of the body. Moreover, the kidneys maintain a constant osmotic potential of the body fluid by controlling the amount of water and mineral salts reabsorbed in the convoluted tubules and the collecting duct. Great fluctuation in the water potential of the cells may kill them (Fox, 1999).

Renal function tests are employed to evaluated the excretory, secretory and osmolar regulation dynamics of the kidneys.

#### *Urea nitrogen*

Urea is a nonprotein nitrogenous compound synthesized by liver from ammonia that is absorbed from the intestine or generated by endogenous protein catabolism. It is freely filtered through glomerulus and excreted in urine. However, some urea is passively reabsorbed with water in the proximal tubule. Blood urea analysis involves measurement of nitrogen, it is expressed as blood urea nitrogen.

It reflects the balance between production and excretion of urea. Changes in protein intake, fluid balance, liver functions and renal excretion will affect the urea nitrogen level (Lum and Leal-Khouri, 1989).

#### *Creatinine*

Creatinine is the end product of creatine metabolism. It is an ideal substance for determining the renal clearance, because a fairly constant quantity is produced

within the body.

With the measurements of serum creatinine and urine creatinine levels, creatinine clearance can be obtained (Walmsley and White, 1994). The term 'clearance' refers to the relationship between the renal excretory mechanisms and the circulation blood levels of the materials to be excreted. Clearance refers the overall efficiency of glomerular functioning.

Creatinine clearance is a sensitive indicator of glomerular function because those factors affecting the clearance are primarily due to alterations in renal function. These factors include the number of functioning nephrons, the efficiency with which they function, and the amount of blood entering the nephrons. Renal disease is the major cause of the reduction in the clearance value. Thus, this measurement is a more sensitive indicator of renal function than the urea nitrogen (Cella and Watson, 1989).

## 1.8 Toxicology

Toxicology encompasses the study of the adverse effects of chemical and physical agents on living organisms and groups of organisms. It assesses the probability of hazards caused by such effects. Also, it estimates the results of these effects on individuals, populations, and ecosystems. Generally, toxicological studies deal with adverse effects ranging from acute to long-term (Schiefer *et al.*, 1997).

### 1.8.1 Acute toxicity test

The acute toxicity test is the first biological experiment done with the majority of compounds synthesized by the chemists or discovered in the environment (Zbinden, 1984). It often provides a wealth of information which can greatly influence the fate of a new chemical or a food substance. The results of acute toxicity tests are used as guidance for dose selection and design of further toxicological experiments.

The acute toxicity of a chemical is its ability to cause either local damage (e.g. to skin or eyes) or systemic damage (affecting the body as a whole), as a result of one exposure to a relatively large amount of that substance (Schiefer *et al.*, 1997).

There are two types of parameters for the acute toxicity study: lethal and non-lethal. $LD_{50}$ is a term commonly used to describe one type of acute toxicity. LD means "lethal dose", and the subscript 50 means that dose of toxicant which is acutely lethal to 50% of test organisms under controlled laboratory conditions. The smaller the $LD_{50}$, the greater the toxicity. Conversely, the larger the $LD_{50}$, the lower the toxicity. Mostly, the animals are closely observed for 14 days, and all adverse effects and deaths are recorded during this time. Besides, non-lethal parameters should also be taken into consideration in evaluating the acute toxicity if the animals

still survive after the 14-day test period. Histopathological examination of the tissues would be a source of understanding the acute toxic effect of the substances for the investigation of the toxicants (Chan *et al.*, 1992; Schiefer *et al.*, 1997).

### 1.8.2 Chronic toxicity test

Chronic toxicity of a chemical or food substance is its ability to cause damage as a result of repeated exposure to relatively small amounts over a prolonged time period. The doses used in chronic toxicity tests are usually lower than those in the acute toxicity tests. This type of toxicity study at low dose exposure offers information on the cumulative effects of the toxicants (Chan *et al.*, 1992). The chronic study examines adverse effects other than death. This may include adverse effects on behavior, organs, blood and urine (Schiefer *et al.*, 1997).

## 1.9 Objective

The objective of this research is to investigate the potential use of CP as a natural food colorant. The overall working hypothesis of this project is that CP is a safe natural pigment which can be applied on food items. Two major properties of a compound to serve such purpose include the stability of the compound and the safety of the compound for consumption. The thermal stability and light stability of CP will therefore be assessed. In addition to these properties, the antioxidative activity found associated with the CP preparation will also be studied as such activity may also attribute to CP's application in food items as a natural preservative (an antioxidant). For the safety evaluation of CP, both acute and chronic toxicity tests using experimental animals will be conducted. Genotoxicity of CP will be determined by the Mutatox test, a mutagenicity test based on the priniciple of the Ames test.

# Chapter 2 Materials and Methods

## 2.1 Plant Materials

*Castanea mollissima* nuts were purchased from a local market in Yuen Long. The shells were peeled manually and kept at 4°C prior to extraction. (Figure 2.1).

## 2.2 Sample preparation

For the extraction of CP, 1000 g Chinese chestnut shells were boiled in 6 L distilled water for five hours. The extract was filtered to remove the residue. It was then concentrated by a rotary-evaporator (Büchi, series RE111) to a small volume.

In the purification process, solvent extraction by dichloromethane and ethyl acetate were performed. The concentrated pigment solution (800 ml) and dichloromethane were mixed in 1:1 ratio in a 2 L separating funnel and extracted three times. The upper aqueous layer, containing the pigment, was again, extracted with ethyl acetate, in 1:1 ratio in a separating funnel and extracted three times. After phase separation, the lower brown aqueous layer was collected and lyophilized to a dry powder, which was about 30 g and stored in a desiccator.

Over 5,000 g purified CP were prepared. The yield was about 1.5%.

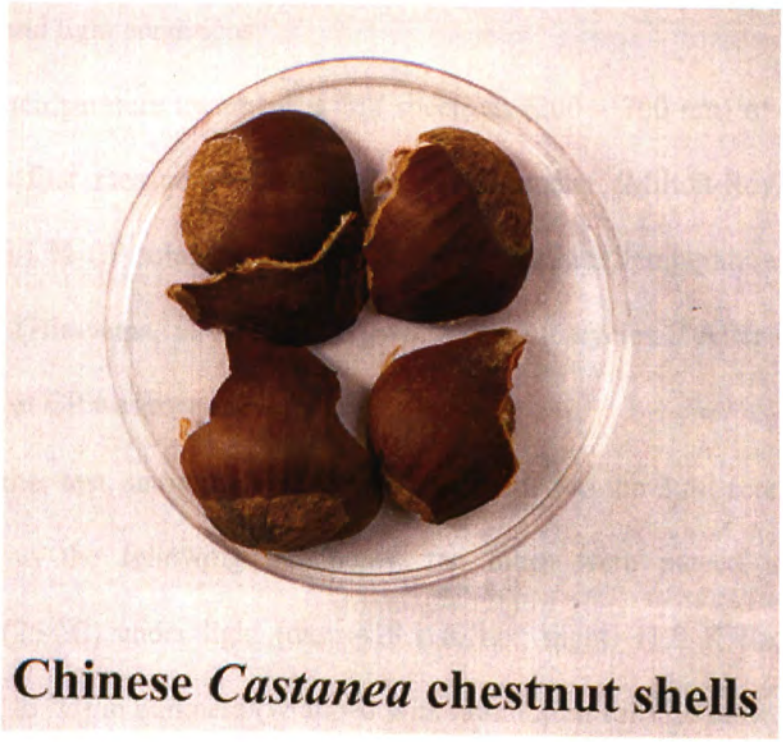



**Figure 2.1 The shells of *Castanea mollissima*.**

## 2.3 Pigment characterization

### 2.3.1 Stability test

In order to observe the stability of CP, the pigment was tested under different temperature and light conditions.

In high temperature treatment, a full spectrum (200 – 700 nm) of a 0.1 % CP solution was first measured with the spectrophotometer (Milton-Roy, Spectronic 601). The 0.1 % CP solution was then treated under high temperature (121 °C) in an autoclave (Hirayama, HA-300P) for two 20-minute cycles. After autoclaving, the spectrum of CP was measured again.

For another test, samples of 0.1 % CP were added to the 2 ml screw-cap tubes and subject to the following treatments: the tubes were placed at (1) room temperature (25 °C) under light (day: 418.1 K lux; night: 11.9 K lux); (2) room temperature (25 °C) in darkness (wrapped with aluminium foil); (3) low temperature (4 °C) in darkness (wrapped with aluminium foil). The experiment duration was 12 months.

The spectrum and absorbance at 400 nm of the samples from each treatment were determined once a month continuously throughout a year.

### 2.3.2 HPLC separation of CP

Reversed phase-HPLC was used in the separation of CP. The analytical system included the Hewlett Packard (HP) series 1100 liquid chromatograph equipped with a HP 1100 photodiode array detector and a HP 1100 computer system. This system was connected with a guard column (Eclipse XDB-C18, 4.6 mm id x 12.5 mm, 5 micron) and a separating column (Eclipse XDB-C18, 4.6 mm id x 150 mm, 5 micron).

A 1% CP solution was first filtered with a minipore filter (0.2 μm) and then injected into the column. The injection volume was 100 μl. The solvent system was (A) 100% ultra pure water and (B) 100% acetonitrile. A linear gradient of 0 to 3 % solvent B was set within 35 minutes. The flow rate was 1 ml/min. The chromatogram was acquired at 400 nm.

Fractions were collected at 1-minute intervals in order to identify the peak responsible for the CP. The collected fractions were dried with the use of a speed-vac (Savant, SPD111V) and then dissolved in methanol.

### 2.3.3 Determination of antioxidative activity with the DPPH$^{\bullet}$ scavenging method

The DPPH$^{\bullet}$ scavenging method was used to determine the free radical scavenging of CP (Brand-Williams *et al.*, 1995). A 0.1% CP methanolic solution (500 μl) was mixed quickly with a $6x10^{-5}$ M methanolic DPPH$^{\bullet}$ solution (2 ml) in a 3 ml cuvette. The methanolic DPPH$^{\bullet}$ solution was freshly prepared. Absolute methanol was used as a control.

In addition, the dried HPLC fractions were dissolved in methanol to make a concentration of 0.1% CP and was then tested in the DPPH$^{\bullet}$ scavenging method.

Antioxidant standards including BHA (20 mM, 50 mM and 100 mM) and BHT (50 mM and 100 mM) were used as references. A spectrophotometer with wavelength set at 515 nm (Milton-Roy, Spectronic 601) was used to read the samples continuously at half-minute intervals for 5 minutes.

The reaction kinetic was plotted for the samples and standards. Each measurement was done in triplicates. The percentage discoloration on $DPPH^{\bullet}$ at the first 5 minutes was calculated by the following formulae (Yen and Duh, 1994):

$$\% \text{ Discoloration } (t = 5) = \frac{A(0) - A(t)}{A(0)} \times 100$$

where $A(0)$ is the absorbance of the mixture at time = 0 minute;

$A(t)$ is the absorbance of the mixture at time = 5 minutes.

## 2.4 Microtox® test

In the present study, the test was carried out with an Azur Toxicity M500 Analyzer (USA) following the test procedures outlined in the Microtox® Manual (Azur Environmental, 1995). All reagents and solutions were purchased from Azur Environmental. Zinc sulfate standard ($ZnSO_4.7H_2O$) was used to check the performance of the complete test system (e.g. analyzer, reagent, diluent and reconstitution solution). In the Screening Test, a sample of CP was first diluted to different concentrations by adding diluent. A suitable concentration of CP was selected to do the Basic Test. It was done in triplicates and osmotic adjustment was performed. EC50s at 5, 15 and 30 minutes and 95% confidence intervals were calculated by the software MTX7 (Azur Environmental, USA). EC50 defines an effective concentration that can have a sub-lethal effect in 50% individuals in the test population. As all the CP samples showed distinct colors, the Color Correction Protocol was performed.

## 2.5 Mutatox® test

Mutatox® test was carried out with an Azur Toxicity M500 Analyzer (USA) following the test procedures outlined in the Mutatox® Manual (Azur Environmental, 1995). All reagents and solutions were purchased from Azur Environmental. Mutatox® test measures the light output at 16, 20 and 24 hours of incubation at 27°C for the optimum growth temperature of a special dark mutant of luminescent bacteria (*V. fischeri*, strain M169).

Toxicity from a test sample can interfere the Mutatox® test system. If the test sample concentration is highly toxic, the cells cannot express any mutagenic effects from the sample. Hence, all samples were measured at its EC50 for comparison and it was useful to select the test sample dilution which gave the greatest response ratio (highest light level at a sample dilution to the appropriate control light level).

Suspected mutagenic agents are defined as those samples which induce the increase in light levels to at least two times the average control reading in at least two consecutive dilution cuvettes (Azur Environmental, 1995). The requirement for at least two positive dilutions avoids scoring a sample positive which may be a spurious result.

## 2.6 Acute Toxicity Test

### 2.6.1 Animals

Sprague-Dawley (SD) male rats of 200 – 230 g from the same colony were supplied from the Laboratory Animal Services Center, The Chinese University of Hong Kong. Mature rats, 8 weeks of age, were used in order to avoid the variability in white cell counts present in immature rats (Turton *et al.*, 1989; Wolford *et al.*, 1987) (Figure 2.2).

### 2.6.2 Housing and maintenance

The animals were housed individually in suspended wire-bottom cages (Figure 2.3). The wire mesh floors in this design of cage allow excrement to fall to absorbant papers underneath the cages. This minimized the need to disturb the cage occupants while changing papers. It had been reported that the heart rate, haemoglobin, plasma protein content, and serum glucose, pyruvate, and lactate concentrations in rats were elevated after cage movement to a table (Gartner *et al.*, 1980).

The rats were maintained under the conditions of 22 – 25 °C with a 12-hour light-dark cycle and a relative humidity of 50 ± 10%. They were supplied with standard animal chow (PicoLab Rodent Diet 20, PMI Nutrition International, Inc., USA) *ad libitum*. Test solutions were also provided *ad libitum* from clear plastic bottles fitted with stainless steel sipper tubes with ball valves. Acclimation period was seven days prior to assay.

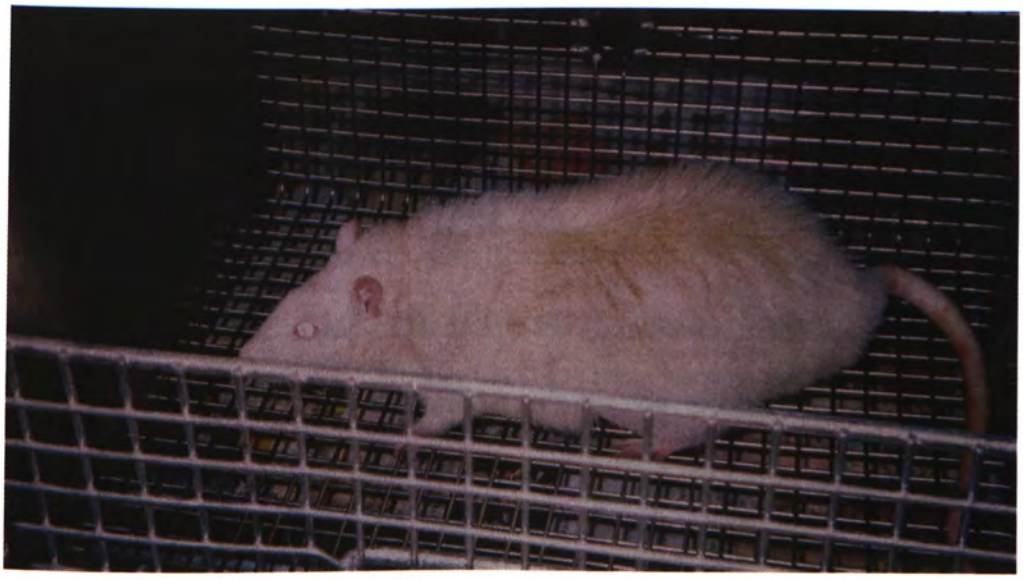


**Figure 2.2 A SD male rat of an acute treatment of CP.**

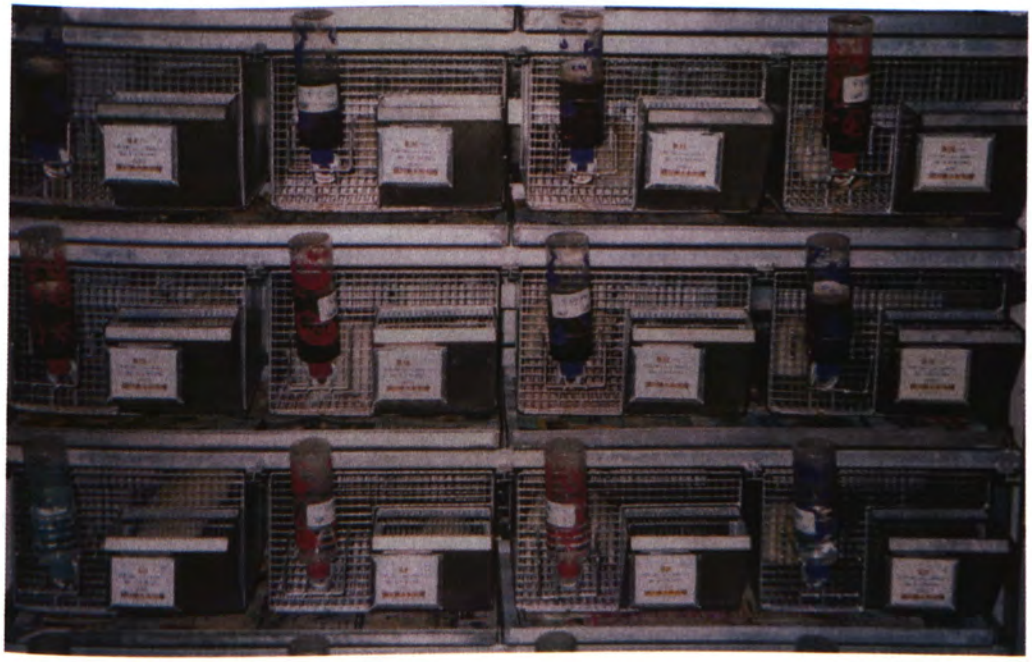


**Figure 2.3 The experimental SD rats were housed in suspended wire-bottom cages.**

### 2.6.3 Experimental design

The experimental period was 14 days. Male SD rats were randomly divided into two groups of eight rats. Group 1 was the control group, provided with distilled water and did not receive any treatment during the course of the experiment. Group 2 was the test group, provided with 8% CP solution in distilled water.

During the experimental period, all rats were observed twice daily and moribund or dead animals noted. Body weights, food consumption and fluid intake, which was determined by weighing the water bottles, were recorded three times a week.

At the termination of the experiment, blood samples and 24-hour urine samples were taken after an overnight fast. The rats were anesthetized by inhaling diethyl ether. Haematology, blood chemistry and urinalysis tests were done (Table 2.1). The blood was collected through the vena cava. A small part of blood, 1 ml was used in the test of haematology while the remaining blood, 5-8 ml was then left at room temperature for about 2 hours to allow complete clotting. After centrifugation at 3000 g for 15 minutes, serum, the clear supernatant was then pipetted into a clean micro-centrifuge tube and stored at 4°C and –70°C until further analysis in blood chemistry. At necropsy, weights of thymus, spleen, liver, kidneys, testes, heart, brain and caecal content were determined.

**Table 2.1 Clinical pathology tests conducted in rat toxicity studies with CP.**

| | |
|---|---|
| **Haematology** | |
| Haemoglobin* | Haematocrit |
| Mean corpuscular volume* | Red blood cell counts |
| Mean corpuscular haemoglobin* | White blood cell counts, total and differential |
| Mean corpuscular haemoglobin concentration* | |
| **Blood chemistry** | |
| Blood urea nitrogen | Total protein |
| Glucose | Albumin |
| Alanine aminotransferase | Globulin |
| Aspartate aminotransferase | Albumin/globulin ratio |
| Total Cholesterol | Immunoglobulins, IgG, IgA and IgM |
| Triglycerides | γ-Glutamyl transpeptidase |
| High-density lipoprotein cholesterol | Calcium |
| Low-density lipoprotein cholesterol | Sodium |
| Total bilirubin | Potassium |
| Alkaline phosphatase | Magnesium |
| Creatinine, serum and urine | Chloride |
| Creatinine clearance | |
| **Urinalysis** | |
| Volume (24-hr) | Bilirubin |
| Physical appearance | Blood |
| pH | Urobilinogen |
| Protein | Specific gravity |
| Glucose | Nitrite |

*Evaluated in the 9-month chronic toxicity test only.

### 2.6.4 Chemicals

Dacie's formol-citrate

Dacie's formol-citrate was a diluting fluid used in the determination of red-cell count. It was prepared by dissolving 3.0 g sodium nitrate and 1.0 ml 40% formaldehyde in 100 ml distilled water. This fluid was isotonic, it contained formaldehyde to fix the cells and prevent the growth the moulds, and citrate to prevent coagulation.

White-cell count reagent

The diluting fluid for white-cell counts completely lysed the red cells without damaging the white cells. It was 2% acetic acid with a little methyl violet added which stained the cells faintly.

Phosphate buffered saline (PBS)

PBS was used in the determinations of serum immunoglobulins. It was prepared by dissolving 156.01 g sodium dihydrogen phosphate, 141.96 g disodium hydrogen phosphate and 58.44 g sodium chloride in 1 L distilled water.

Washing buffer (PBS-T)

PBS-T was used in the determinations of serum immunoglobulins. It was prepared by dissolving 156.01 g sodium dihydrogen phosphate, 141.96 g disodium hydrogen phosphate, 58.44 g sodium chloride and 0.5 ml polyoxyethylene sorbitan monolaurate (Tween 20) (Sigma, P1379) in 1 L distilled water.

Acid buffer

Acid buffer was used to determine serum chloride levels. The composition of which was as follows: polyvinyl alcohol (0.9% w/v), glacial acetic acid (10% w/v), nitric acid (0.64% w/v) and NaCl (0.004% w/v).

10% buffered formalin

Buffered formalin (10%) of pH 7.0 was used to fix organs in histological study. It was prepared by dissolving 8.5 g sodium chloride, 4.0 g hydrated sodium dihydrogen phosphate, 6.5 g disodium hydrogen phosphate in 100 ml formalin and 900 ml distilled water.

### 2.6.5 Clinical pathology test

#### 2.6.5.1 Haematology

***Determination of total red blood cells (RBC)***

After the blood was collected, immediately, 5 µl fresh blood was mixed well with 1 ml Dacie's formol-citrate giving a dilution of 1 in 200. About 8 µl diluted blood was then pipetted into a Neubauer counting chamber. After leaving the cells to settle for 2 minutes in a moist chamber, the cells were counted under the 200x microscope magnification (Figure 2.4).

***Determination of haematocrit or packed cell volume (PCV)***

A glass capillary tube was almost filled with non-clotted whole blood and was placed in a microhaematocrit centrifuge (Hettich, 201424). After centrifugation for 3 minutes, the capillary tube (Figure 2.5) was removed and the value of haematocrit was determined by this formulae: Haematocrit (%) = b/(a+b) x 100%.

***Determination of total white blood cells***

Immediately after collected, 50 µl fresh blood was mixed well with 0.95 ml of white-cell count reagent giving a dilution of 1 in 20. About 8 µl diluted blood was then pipetted into a Neubauer counting chamber. After leaving the cells to settle for 2 minutes in a moist chamber, the cells were counted using the 50x microscope magnification (Figure 2.4).

***Determination of differential white-cell count***

The examination of a stained blood film under the microscope could probably

produce more information than any other single test in haematology. It was therefore important to make and stain a film well, and to examine it systematically. The film was made according to Kirk *et al.* (1986). After drying the film for about two hours, the slide was stained by the differential count staining method (modified from Kirk *et al.*, 1986).

The procedure was as follows: The film was fixed in methanol for 10 minutes and then stained with May-Grunwald stain (Sigma, MG-500) diluted in an equal volume of buffered water for 10 minutes. Afterwards, the film was rinsed briefly with buffered water and stained with Giemsa stain (Sigma, GS-500) diluted in 1 in 10 in buffered water for 30 minutes. Finally, it was rinsed with distilled water and dried in air. The film was then examined under a light microscope (1000x) and different types of white cells could be counted using a 5-unit counter (Danaher Controls, 0149000 – 105).

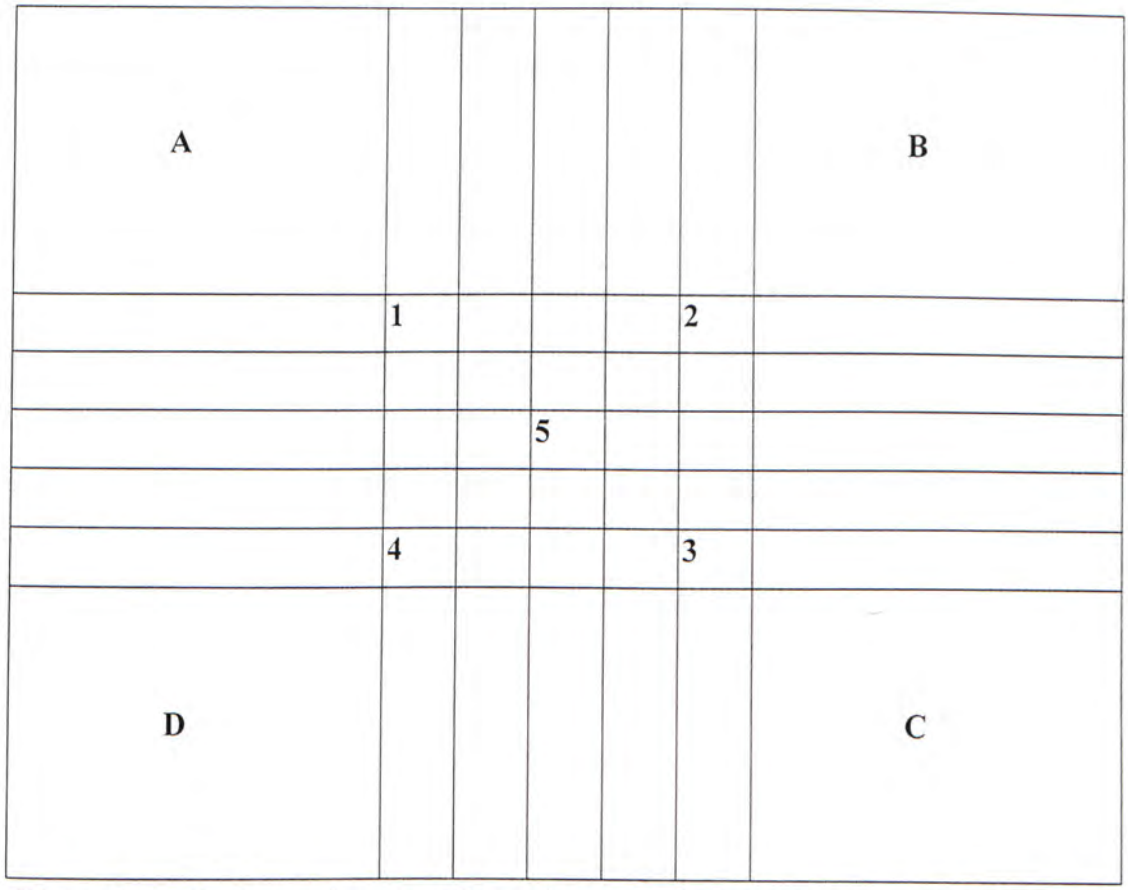



**Figure 2.4 Cell counting procedures for red-cell count squares 1, 2, 3, 4 and 5; for total white-cell count large squares A, B, C and D.**

*Red-cell count:* since there are 25 groups of squares in the central square millimeter, 5 groups are equivalent to 0.2 $mm^2$. If cell count in 5 groups of squares (i.e. 80 small squares) = N. Then, the number of cells in 1 $mm^2$ is N x 5. Since the depth is 0.1 mm, then the number of cells in 1 $mm^3$ is N x 5 x 10. The blood is diluted 1 in 200. Therefore,

The number of red blood cells in 1 $mm^3$ of whole blood

= N x 5 x 10 x 200

= N x 10,000

where N = cell count in 5 groups (1-5) of squares (i.e. 80 small squares)

*Total white-cell count:*

If the number of cells counted in 4 $mm^2$ is N, then the number of cells in 1 $mm^2$ is N/4. Since the depth of the fluid is 0.1 mm and the dilution of blood is 1 in 20. Therefore,

The number of white blood cells in 1 $mm^3$ of whole blood

= N/4 x 10 x 20

= N x 50

where N = total number of cells in four of the larger corner squares (A-D)

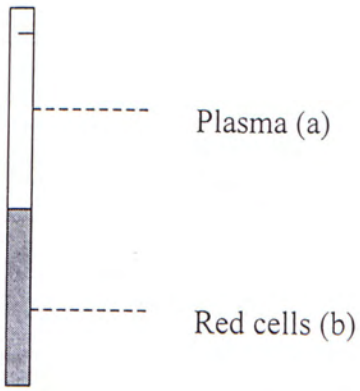



**Figure 2.5 A glass capillary tube used in determination of haematocrit.**

### 2.6.5.2 Blood chemistry

**Determination of serum glucose and lipids**

***Glucose***

Serum glucose levels were measured by a coupled glucose oxidase-peroxidase reaction (Sigma procedure 510). Color-enzyme substrate was prepared freshly by dissolving 1 capsule of glucose oxidase and peroxidase (PGO) enzyme mixture (Sigma, USA) in 100 ml distilled water and adding 1.6 ml of o-Dianisidine (Sigma, USA). One ml of color-enzyme substrate was mixed with 10 µl serum sample or glucose standard (0-100 mg/dL) and a brown color developed after 30 minutes incubation at 37°C water bath. Absorbance was taken at 450 nm. The procedure is based upon the following coupled enzymatic reactions:

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{o-Dianisidine} \xrightarrow{\text{Peroxidase}} \text{Oxidized o-Dianisidine}$$

(colorless) (brown)

***Total cholesterol***

Serum total cholesterol was assayed using the commercial diagnostic kit (Sigma, 352). Enzymatic reactions involved in the procedures are as follows:

$$\text{Cholesterol Esters} + H_2O \xrightarrow[\text{Esterase}]{\text{Cholesterol}} \text{Cholesterol} + \text{Fatty Acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{Oxidase}]{\text{Cholesterol}} \text{Cholest-4-en-3-one} + H_2O_2 + 2H_2O_2 + \text{4-Aminoantipyrine} + \text{p-Hydroxybenzenesulfonate}$$

$$\xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + 4\ H_2O$$

The intensity of the color produced in quinonoimine dye which has an absorbance maximum at 500 nm is directly proportional to the total cholesterol concentration in the sample.

***Triglycerides***

Serum triglycerides was assayed using the commercial diagnostic kit (Sigma, 336). Triglycerides are first hydrolyzed by lipoprotein lipase to glycerol and free fatty acids. The resulting formazan is highly colored and has an absorbance maximum at 500 nm. The intensity of the color produced is directly proportional to the triglycerides concentration of the sample. The enzymatic reactions involved in the assay are as follows:

$$\text{Triglycerides} \xrightarrow[\text{Lipase}]{\text{Lipoprotein}} \text{Glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{G-1-P} + \text{ADP}$$

$$\text{G-1-P} + \text{NAD} \xrightarrow{\text{G-1-PDH}} \text{DAP} + \text{NADH}$$

$$\text{NADH} + \text{INT} \xrightarrow{\text{Diaphorase}} \text{Formazan} + \text{NAD}$$

***High-density lipoprotein (HDL) cholesterol***

Total cholesterol in serum comprises all the cholesterol found in various lipoproteins. Cholesterol is the major component of the low density lipoproteins (LDL) fraction. It is also a minor component of the very low density lipoprotein (VLDL) and high density lipoprotein (HDL) fractions. In this assay, LDL and VLDL were first separated from HDL according to the Sigma procedure 352-4. The subsequent supernatant that contained HDL was then assayed for cholesterol (Sigma, 352).

***Low-density lipoprotein (LDL) cholesterol***

Serum low-density lipoprotein (LDL) cholesterol concentration was calculated by using the formula validated by Friedewald *et al.* (1972):

LDL cholesterol = Total cholesterol – HDL cholesterol – Triglycerides/5

This equation was originally designed for humans, but was also applied in rats and hamsters (Balmer *et al.*, 1996).

## Determination of serum proteins

### *Total protein*

Serum total protein was measured according to the Sigma procedure 541. It was measured based on the biuret method. The copper ions in alkaline biuret reagent, react with peptide bonds of serum proteins to form a purple color with an absorbance maximum at 540 nm. The intensity of the color is proprotional to the total protein concentration.

### *Albumin*

Serum albumin levels were assayed using the commercial diagnostic kit (Sigma, 625). It was based on the principle that serum albumin reacts specifically with bromcresol purple to form a stable blue-purple color complex with an absorption maximum at 600 nm. The intensity of the color is proportional to the serum albumin concentration in the sample.

### *Globulin*

Serum globulin level was the concentration of albumin subtracted by that of total protein.

### *Immunoglobulins (IgG, IgA and IgM)*

Determinations of total serum IgG, IgA and IgM were performed by enzyme–linked immunosorbent assay (ELISA) (Vos *et al.*, 1982).

For IgA and IgM determinations, primary monoclonal mouse anti-rat IgA (Sigma, R0636) and IgM (Sigma, R0886) were used together with secondary anti-mouse IgG peroxidase conjugate (Sigma, A2304).

For IgG determination, anti-rat IgG perxidase conjugate (Sigma, A5795) was

available, and no secondary antibody was needed.

The substrate was 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma, T8665). In each study, a serum pool of the control animals was used as reference serum.

The subsequent steps were shown in the followings:

*Antigen coating:*

Serum samples (100 µl) were mixed with an equal volume of phosphate buffered saline (PBS) and were then added to each well of a 96-well plate (Sigma, M0156). The plate was incubated at 37 °C for 30 minutes. After the incubation process, the coating solution was removed and the wells were washed three times with washing buffer (PBS-T).

*Primary antibody reaction:*

The monoclonal primary antibodies, anti-rat Ig-A (1 in 500 PBS-T), anti-rat IgM (1 in 1,000 PBS-T) and anti-rat IgG (1 in 50,000 PBS-T), 200 µl each, were added to each well. After the incubation for two hours at room temperature, the solution was removed and the wells were washed three times with 200 µl washing buffer.

*Application of secondary antibody (only for IgA and IgM determinations):*

The peroxidase conjugated secondary antibody, anti-mouse IgG was diluted 1 in 50,000 PBS-T. The diluted antibodies (200 µl) were then added to each well. After the incubation for two hours at room temperature, the solution was removed and the wells were washed three times with 200 µl washing buffer.

*Substrate preparation and color development:*

For each microwell, 200 µl of TMB substrate solution was added and the plate was then incubated for 30 minutes at room temperature. After acidification with 100 µl of 0.5 M sulphuric acid stopping solution, a yellow color was produced and the absorbance was read at 450 nm.

***Albumin/Globulin (A/G) ratio***

Serum A/G ratio was calculated by the amount of serum globulin divided by the amount of serum albumin.

**Determination of liver functions**

***Alanine transferase (ALT)***

Serum ALT was reported to be stable for about 1 week when refrigerated and for about 2 days at room temperature (Henry, 1968). ALT loses activity when serum is frozen. Serum ALT levels were assayed by a diagnostic kit (Sigma, 505).

***Aspartate transferase (AST)***

Serum AST was reported to be stable for at least 2 weeks when refrigerated and possibly longer when stored frozen (Henry, 1968). Similar to ALT, serum AST levels were assayed by a diagnostic kit (Sigma, 505). The reactions for ALT and AST are as follows:

$$\text{Alanine} + \alpha\text{-Ketoglutaric acid} \xrightarrow{\text{ALT}} \text{Pyruvic acid} + \text{Glutamic acid}$$

$$\text{Aspartic acid} + \alpha\text{-Ketoglutaric acid} \xrightarrow{\text{AST}} \text{Oxalacetic acid} + \text{Glutamic acid}$$

The oxalacetic or pyruvic acid formed in the above reactions reacts with 2,4-dinitrophenylhydrazine. The color intensity of the resulting phenylhydrazones with a maximum absorbance at 505 nm is proportional to the transaminase activity.

***Alkaline phosphatase (ALP)***

Serum ALP levels were assayed using the commercial diagnostic kit (Sigma, 104). The measurement is based upon the hydrolysis reaction:

$$\underset{\text{(colorless)}}{\text{p-Nitrophenyl phosphate} + H_2O} \xrightarrow{\text{ALP}} \underset{\text{(yellow)}}{\text{p-Nitrophenol} + \text{Inorganic phosphate}}$$

When alkaline, p-nitrophenol is converted to a yellow complex readily measured at 410 nm. The intensity of color formed is proportional to ALP activity.

***γ-glutamyl transpeptidase (GGTP)***

Serum GGTP activities were measured using the commercial diagnostic kit (Sigma, 545). This assay is based on the transfer of the glutamyl group from L-glutamyl-p-nitroanilide to glycylglycine catalyzed by GGTP as follows:

L-glutamyl-p-nitroanilide + Glycylglycine $\xrightarrow{\text{GGTP}}$ p-Nitroaniline + Glutamyl-glycylglycine

p-Nitroaniline + Sodium nitrite $\xrightarrow{\text{Acid}}$ Diazo compound

Diazo compound + Naphthylethylenediamine $\longrightarrow$ Pink azo-dye

The absorbance of the pink azo-dye measured at 540 nm is proportional to GGTP acitivity.

***Total bilirubin***

Serum total bilirubin levels were assayed using the commercial diagnostic kit (Sigma, 552). Both conjugated and unconjugated forms of bilirubin were measured. Determination of serum total bilirubin is generally based on its reaction with diazotized sulfanilic acid to form a colored product which is proportional to the bilirubin concentration and with an absorbance maximum at 540 nm.

**Determination of renal functions**

***Urea nitrogen***

Serum urea nitrogen levels were assayed using the commercial diagnostic kit (Sigma, 535). Urea concentration is directly proportional to intensity of the color (535 nm) produced in the following reaction:

Diacetyl monoxime + Urea ⟶ Pink chromogen + Hydroxylamine

***Creatinine***

Both serum and urine creatinine levels were assayed using the commercial diagnostic kit (Sigma, 555). This assay is based on the following reaction:

Creatinine + Picric acid $\xrightarrow{\text{Alkali}}$ Colored complex

The difference in color intensity measured at 500 nm before and after acidification is proportional to creatinine concentration.

***Creatinine clearance***

Creatinine clearance was calculated by:

Creatinine clearance (ml/min.)

= (Urine creatinine level x 24-hour urine volume) / Serum creatinine level

**Determination of serum electrolytes**

Serum sodium, potassium, calcium and magnesium levels were determined, after approximate dilutions (20 µl serum in ultra-pure water), using a flame photometer (PHF 90D, ISA). Respective standard curves were constructed ($Na^+$: 0-150 mEq/L; $Ca^{2+}$: 0-20 mg/dL; $K^+$: 0-10 mEq/L; $Mg^{2+}$: 0-10 mg/dL).

Serum chloride levels were measured, via titration, using a chloride meter (Corning-eel, 920), conductivity being measured by an electrode after the addition of a 20 µl sample/standard directly into an acid buffer.

### 2.6.5.3 Urinalysis

***Urine volume***

A 24-hour urine sample was collected with the use of a metabolic cage (Figure 2.6) one day before the end of the experiment. One rat was put inside the cage and the cage was placed on top of a funnel device so that urine falling on the sides of the funnel would go to the side arm and then came to the collecting jar, while the faeces pellets dropped into another collecting jar.

***Urinalysis dip-and-read test strips***

Urinalysis dip-and-read test strips (SELF-STIK, Korea) provide tests for, qualitatlively and semiquantitatively, urobilinogen (0-2 mg/dL), glucose (0-400 mg/dL), ketones (0-80 mg/dL), bilirubin (0-3 mg/dL), protein (0-1000 mg/dL), nitrite, pH (5-9), blood, specific gravity (1.000-1.030) and leukocytes. After dipping a test strip completely for no more than 1 second in fresh, well-mixed, and uncentrifuged urine specimen, the test results were compared with the color chart on the bottle label in good light within a proper reading time (30-60 seconds).

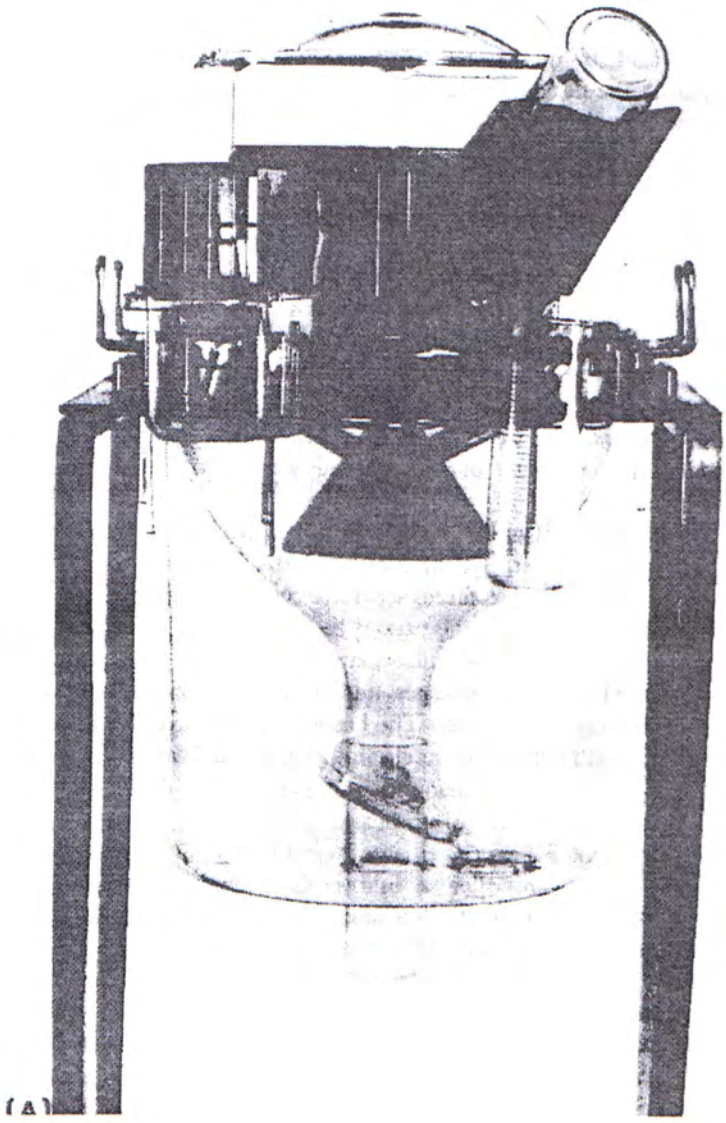


**Figure 2.6 A metabolic cage used for the collection of urine sample.**

### 2.6.6 Histological study

Left lobe of the liver, kidney and thymus were all cut into small pieces and fixed individually in 10% buffered formalin for at least two days. The fixed tissues were then dehydrated in a series of ethanol and xylene, embedded in paraffin and cut into sections of 5 μm in thickness by using a microtome (Leica, 2035). The sections were then mounted on slide, stained by hematoxylin and eosin (H&E) and observed under light microscope after mounting with Canada Balsam. The detail procedures for dehydration, embedding and staining were presented in Table 2.2.

### 2.6.7 Statistical analysis

All results were expressed as arithmetical mean ± standard deviation (SD). Statistical differences between groups were compared by one way Analysis of Variance (ANOVA) (by Excel) followed by Tukey test (by SPSS), with a significance level of 95% unless specified.

**Table 2.2. Procedure for tissue preparation for light microscopic study.**

| Procedure | | Reagent | Time |
|---|---|---|---|
| **Fixing** | | 10% buffered formalin | At least 2 days |
| **Processing:** | *Dehydration* | 50% EtOH | 1.5 hours |
| | | 70% EtOH | 1.5 hours |
| | | 85% EtOH | 1.5 hours |
| | | 95% EtOH | 2 hours |
| | | Absolute EtOH | 45 min |
| | | Absolute EtOH | 45 min |
| | | Absolute EtOH | 45 min |
| | *Clearing* | Xylene:EtOH (1:1) | 45 min |
| | | Xylene | 45 min |
| | | Xylene | 45 min |
| | *Infiltration* | Paraffin wax | 45 min |
| | *Infiltration* | Paraffin wax | 45 min |
| **Embedding:** | Tissues were embedded in paraffin wax | | |
| **Sectioning:** | Tissues were cut into sections of 5 μm in thickness | | |
| **Staining:** | *Dewax* | Xylene | 5 min |
| | | Xylene | 5 min |
| | *Hydration* | Absolute EtOH | 1 min |
| | | 95% EtOH | 1 min |
| | | 70% EtOH | 1 min |
| | | 50% EtOH | 1 min |
| | | 30% EtOH | 1 min |
| | | Running tap water | 1 min |
| | *Staining* | Mayer's hematoxylin | 20 min |
| | | Running tap water | 1 min |
| | | Acid EtOH | ~ 1-5 sec |
| | | Running tap water | 1 min |
| | | Scott's tap water | 2 min |
| | | Running tap water | 1 min |
| | | 0.5% aqueous Eosin | 1-2 min |
| | | Running tap water | 1 min |
| | | 70% EtOH | As short as possible |
| | | 95% EtOH | As short as possible |
| | | Absolute EtOH | 2 min |
| | | Absolute EtOH | 2 min |
| | | Xylene:EtOH (1:1) | 2 min |
| | | Xylene | 2 min |
| | | Xylene | 2 min |
| **Mounting:** | Slides were mounted with Canada Balsam | | |

## 2.7 Chronic Toxicity Test

### 2.7.1 Animals

Sprague-Dawley (SD) male weaning rats of 40 – 50 g from the same colony were supplied from the Laboratory Animal Services Center, The Chinese University of Hong Kong.

### 2.7.2 Housing and maintenances

The arrangements were the same as those described in *Section 2.6.2* of *Materials and Methods*.

### 2.7.3 Experimental design

In this study, 6-month and 9-month chronic treatments were carried out independently. In each chronic treatment, male SD rats were randomly divided into groups of eight. Group 1 was the control group, provided with distilled water and did not receive any treatment during the course of experiment. Group 2 and group 3 were the treatment groups, provided respectively with 0.25% and 1% CP solutions in distilled water. During the experimental period, all rats were observed twice daily and moribund or dead animals, if present, were noted. Body weights, food consumption and fluid intake were recorded weekly.

In the 6-month chronic treatment, after carrying out the test for three months, a small volume (1.5 ml) of blood samples were obtained from the tail of each rat. The blood samples were analyzed by the tests of haematology and blood chemistry.

At the end of the experiment, blood samples and 24-hour urine samples were taken after the overnight fast. The rats were anesthetized by inhaling diethyl ether. The blood was collected via the vena cava. Haematology, blood chemistry and

urinalysis tests were done (Table 2.1). At necropsy, macroscopic abnormalities were recorded; weights of thymus, spleen, liver, kidneys, testes, heart, brain and caecal content were taken.

### 2.7.4 Chemicals

The details were the same as those described in *Section 2.6.4* of *Materials and Methods* with the addition of the following:

Drabkin's solution

Drabkin's solution was used to determine haemoglobin concentration. It was prepared by dissolving 200 mg potassium ferricyanide and 50 mg potassium cyanide in 1 L distilled water. The solution had a pH of 9.6 and was clear.

### 2.7.5 Clinical pathology test

#### 2.7.5.1 Haematology

The details were the same as those described in *Section 2.6.5.1* of *Materials and Methods* with the addition of the followings:

***Determination of haemoglobin (Hb) concentration***

Immediately after collection, 5 µl fresh blood was mixed well with 1 ml Drabkin's solution and then left for 10 minutes. A spectrophotometer with wavelength set at 540 nm was used to read the samples against a standard (Sigma, 525-18), using the Drabkin's solution as a blank.

***Determination of mean cell volume (MCV)***

MCV is the average volume in cubic micrometers of a single red cell. It is calculated by the following formulae:

MCV ($\mu m^3$) = [PCV (%) x 10]/RBC (millions)

***Determination of mean cell haemoglobin (MCH)***

MCH is the average weight of haemoglobin in prcograms (pg) in a single red cell. It is calculated by the following formulae:

MCH (pg) = [Hb (g/dL) x 10]/RBC (millions/$mm^3$)

***Determination of mean cell haemoglobin concentration (MCHC)***

MCHC is a good intdex of hypochromasia present. It is calculated by the following formulae:

MCHC (%) = [Hb (g/dL) x 100]/PCV (%)

#### 2.7.5.2 Blood chemistry

The details were the same as those described in *Section 2.6.5.2* of *Materials and Methods*.

#### 2.7.5.3 Urinalysis

The details were the same as those described in *Section 2.6.5.3* of *Materials and Methods*.

### 2.7.6 Histological study

Selected tissues including liver, kidney and thymus, also testis for the 9-month chronic test, were fixed in 10% buffered formalin for histopathological examination. The detail procedures for dehydration, embedding and staining were presented in Appendix B.

### 2.7.7 Statistical analysis

All results were expressed as arithmetical mean ± standard deviation (SD). Statistical differences between groups were compared by one way Analysis of Variance (ANOVA) (by Excel) followed by Tukey test (by SPSS), with a significance level of 95% unless specified.

# Chapter 3 Results

## 3.1 Pigment characterization

### 3.1.1 Stability test

The spectrum of 0.1 % purified CP was shown in Figure 3.1a. Application of a high temperature by autoclaving did not change the color intensity of CP, and its spectrum remained unchanged. Effect of storage time on the absorbance of 0.1 % CP of the three treatments was shown in Figure 3.2. During the one year of storage, the absorbance reading at 400 nm was not significantly changed ($p<0.05$).

### 3.1.2 HPLC separation of CP

The aqueous extract of 1 % CP was separated into different fractions according to their polarities. The reversed phase HPLC profile of 1 % CP was shown in Figure 3.3. There was a major peak found at retention time of 19 min. The fraction collected from this peak was found to be light brown in color, indicating the presence of CP. The spectrum of CP of the HPLC-19 min. fraction was shown in Figure 3.1b.

### 3.1.3 Antioxidative activities of CP preparations

$DPPH^{\bullet}$ scavenging method was used to determine and compare the free radical scavenging abilities of two CP preparations, namely the 0.1 % CP methanolic solution and the HPLC fraction collected as described in Section 3.1.2; and two synthetic antioxidants, BHA and BHT.

The reaction kinetic was plotted in Figure 3.4. The steeper the slope indicates the stronger the free radical scavenging ability of the sample. The descending order of free radical scavenging abilities of the samples and standards was as follows: 100 mM BHA > 50 mM BHA > 25 mM BHA > 0.1 % CP > 100 mM BHT > 50 mM BHT > HPLC fraction.

Moreover, the percentage discoloration of $DPPH^{\bullet}$ at the first 5 minutes was shown in Figure 3.5. The percentage discoloration was the greatest for 0.1 % CP (95.79±8.15 %) and the lowerest for the HPLC fraction (26.71±1.25 %).

## 3.2 Microtox® test

The toxicity of CP in Microtox® test was shown in Figure 3.6. The light level implies the survival of the bacteria in the tested sample. Therefore, the higher the light level means the lower the toxicity of the tested sample. Figure 3.6 showed that EC50 of the purified CP was much higher than that of the crude one, indicating the toxicity of CP was reduced as a result of purification to remove toxic impurities by organic solvents including dichloromethane and ethyl acetate.

## 3.3 Mutatox® test

Mutatox test was used to detect the presence of mutagenic agents in tested samples. The results on the mutagencity of CP were shown in Table 3.1. In this test, the mean media control light level reading was 2. If a sample had a light level at least two times the average control reading (i.e. 4) in at least two consecutive cuvettes, this sample was a suspected mutagen. As all of the cuvettes of CP had a light level reading below 4, so it was not considered as a mutagenic agent.

## 3.4 Acute toxicity test

### 3.4.1 Growth rate

The increases in body weights were shown in Figure 3.7. All rats were growing steadily. There was no significant difference ($p<0.05$) between the two groups and the final body weights were about 350 g.

### 3.4.2 Food and fluid consumption

For the food and fluid consumption, both groups had no significant difference ($p<0.05$). The food intake per rat was about 25 g a day while the fluid consumption per rat was 32 ml a day. Table 3.2 shows the consumption of drinking fluid and the corresponding CP intake of each group, which has no significant difference ($p<0.05$) between the 2 groups. The tolerated dose of CP per day in the treatment group was 7143±109 mg/kg body weight, which is a very high dosage to the rats.

### 3.4.3 Organ-weight

Relative organ weights of thymus, kidneys, liver, heart, spleen, brain and testes were given in Figure 3.8. For the 8% CP-exposed rats, their relative testes weights (left testis: 0.46±0.03 % body weight; right testis: 0.47±0.03 % body weight) were significantly higher than the control (left testis: 0.42±0.04 % body weight; right testis: 0.42±0.04 % body weight) only at $p<0.05$. There was no significant difference at $p<0.01$.

Relative caecal contents of control and 8% CP-exposed rats were shown in

Figure 3.9. They were obtained by the difference between the weights of full caecums and the empty caecums. Application of a high dosage, 8% CP to the treatment group, could significantly increase their caecal contents from 0.21±0.13 % body weight (control) to 0.72±0.41 % body weight (treatment) ($p<0.05$).

### 3.4.4. Clinical pathology tests

#### 3.4.4.1 Haematology

***Red blood cell count***

Both the control group (7.02±0.69 x $10^6$/$mm^3$) and the treated group (6.78±0.55 x $10^6$/$mm^3$) had the red blood cell count within the reference range of 6.7–9.0 x $10^6$/$mm^3$ (Gad and Chengelis, 1992). There was no significant difference ($p<0.05$) in both groups (Figure 3.10a).

***Haematocrit***

The reference range of haematocrit is 39-55 % (Gad and Chengelis, 1992). All rats, with (52.89±1.59 %) or without treatment (52.50±2.85 %) had their haematocrits within the range with no significant difference ($p<0.05$) (Figure 3.10b).

***Total white blood cell count***

The reference range of total white blood cell count is 3.0–14.5 x $10^3$/$mm^3$ (Gad and Chengelis, 1992). Figure 3.10c showed that there was no significant difference ($p<0.05$) between the control and the treatment groups, and their total white-cell count were 9.75±4.70 x $10^3$/$mm^3$ and 8.70±3.45 x $10^3$/$mm^3$ respectively.

***Differential white blood cell count***

The composition of five types of white blood cells, namely neutrophils, basophils, lymphocytes, monocytes and eosinophils were shown in Figure 3.11. Both the control and treatment groups had similar results. Lymphocytes (~ 55%) and neutrophils (~ 40%) occupied the majority of white blood cells, while monocytes

(~ 5%) and eosinophils (~ 5%) were in a little amount. Basophils were not detected.

All data were within the reference range where the neutrophils is 11.5–41.6 %, basophils is 0 %, lymphocytes is 43.0–79.5 %, monocytes is 0–4 %, eosinophils is 0–4 % (Gad and Chengelis , 1992).

### 3.4.4.2 Blood chemistry

**Glucose and lipids**

***Glucose***

The reference range of fasting blood glucose concentration in rats is 92–192 mg/dL (Minematsu *et al.*, 1995). The rats in the treatment group had a comparative lower than normal blood glucose level at 82.06±18.05 mg/dL while the control group had a normal level at 105.05±29.66 mg/dL. Despite this, there was no significant difference ($p<0.05$) between the two groups (Figure 3.12).

***Total cholesterol***

The reference range of serum total cholesterol concentration in rats is 53.5–112.9 mg/dL (Minematsu *et al.*, 1995). Both the control and treatment groups had similar normal levels, which were 57.20±5.11 mg/dL and 56.99±8.84 mg/dL respectively (Figure 3.13a), with no significant difference ($p<0.05$).

***Triglycerides***

The reference range of serum triglycerides concentration is 31.3–115.7 mg/dL (Minematsu *et al.*, 1995). Triglycerides concentration of rats in the treatment group was found to be 34.63±3.59 mg/dL while the control one was 40.23±5.76 mg/dL (Figure 3.13b), well within the reference range, with no significant difference ($p<0.05$).

***High-density lipoprotein (HDL) cholesterol***

Figure 3.13c showed the effect of 8% CP solution on the HDL cholesterol of SD rats. HDL cholesterol concentrations of the control and treatment groups were

found to be 30.86±4.39 mg/dL and 31.71±6.04 mg/dL respectively, with no significant difference ($p<0.05$).

***Low-density lipoprotein (LDL) cholesterol***

The rats drinking 8% CP solution had a LDL cholesterol level of 16.33±5.75 mg/dL in serum while the control group had 20.88±9.55 mg/dL, with no significant difference ($p<0.05$) (Figure 3.13d).

**Proteins**

***Total protein***

The reference range of serum total protein in rats is 4.85–5.73 g/dL (Minematsu *et al.*, 1995). The rats in control and treatment groups had a total protein level of 6.02±0.58 g/dL and 5.73±0.37 g/dL respectively (Figure 3.14a). There was no significant difference ($p<0.05$) between the two groups although the protein level of the control rats was slightly above the reference range.

***Albumin***

The rats in the control and treatment groups had an albumin concentration of 1.08±0.20 g/dL and 1.01±0.12 g/dL respectively. No significant difference was found ($p<0.05$) (Figure 3.14b).

***Globulin***

The rats in the control and treatment groups had a globulin concentration of 4.95±0.45 g/dL and 4.72±0.27 g/dL respectively. No significant difference was found ($p<0.05$) (Figure 3.14c).

***Albumin/globulin (A/G) ratio***

The rats in the control and treatment groups had an A/G ratio of 0.22±0.04 and 0.21±0.02 respectively. No significant difference was found ($p<0.05$) (Figure 3.14d).

***Immunoglobulins (IgG, IgA and IgM)***

Results of total serum IgG, IgA and IgM determinations were shown in Figures 3.15. There was no significant difference ($p<0.05$) between the two groups for the three immunoglobulins measured.

**Liver function**

***Aspartate transferase (AST)***

The serum AST level has decreased significantly ($p<0.05$) from 70.59±10.94 U/L (control) to 50.09±5.49 U/L (treatment) (Figure 3.16), which is also lower than the reference range of 55.6–94.1 U/L (Minematsu *et al.*, 1995).

***Alanine transferase (ALT)***

The serum ALT level was not altered, being 11.17±1.78 U/L for the control group and 9.37±1.9 U/L for the treatment group (Figure 3.16). These levels, however, were lower than the reference range of 23.1–42.7 U/L (Minematsu *et al.*, 1995).

***Alkaline phosphatase (ALP)***

Application of 8% CP solution was having no significant effects ($p<0.05$) to the ALP levels of the rats. The rats in the control and treatment groups had

91.06±12.97 U/L and 97.92±14.95 U/L respectively (Figure 3.17a). These fell within the reference range of 56.8–128.0 U/L (Gad and Chengelis, 1992).

***γ-glutamyl transpeptidase (GGTP)***

The treated rats drinking 8% CP solution exhibited a significant reduction ($p<0.05$) in serum GGTP level. GGTP levels of the control and treatment groups were 0.46±0.11 Units/mL and 0.24±0.09 Units/mL respectively (Figure 3.17b).

***Total bilirubin***

The reference range of serum total bilirubin level in rats is 0.093–0.189 mg/dL (Minematsu *et al.*, 1995). Serum total bilirubin levels of the control and treatment groups were 0.14±0.04 mg/dL and 0.13±0.37 mg/dL respectively (Figure 3.17c). No significant difference ($p<0.05$) was found between the two groups.

**Renal function**

***Urea nitrogen***

Blood urea nitrogen concentrations of the control (12.42±1.47 mg/dL) and treatment group (13.24±1.70 mg/dL) had no significant difference ($p<0.05$) and were within the reference range, 11.9–18.8 mg/dL (Minematsu *et al.*, 1995) (Figure 3.18).

***Creatinine***

Both serum creatinine and urine creatinine levels were measured (Figures 3.19). Serum creatinine levels of both control (0.72±0.09 mg/dL) and treatment groups (0.73±0.07 mg/dL), were high when compared with the reference range of 0.267-0.351 mg/dL (Minematsu *et al.*, 1995), although no significant difference

($p<0.05$) was found.

For the rats drinking the 8% CP solution, urine creatinine levels of control and treatment groups were 64.61±21.97 mg/dL and 56.37±14.17 mg/dL, respectively, which had no significant difference ($p<0.05$).

***Creatinine clearance***

With the measurement of serum creatinine, urine creatinine levels and 24-hour urine volumes (Figure 3.19c), creatinine clearances of rats were determined. Creatinine clearances of the control and treatment groups, which had no significant difference ($p<0.05$), were determined to be 0.80±0.29 ml/min. and 0.76±0.15 ml/min. respectively (Figure 3.34). The clearances fell within the reference value of creatinine clearance in rats which is 1.2 ml/min. (Waynforth and Flecknell, 1992).

**Electrolytes**

Levels of serum sodium, chloride, potassium, calcium and magnesium ions were shown in Figures 3.20-3.21. No significant difference ($p<0.05$) was found.

Sodium ion concentration of control and treatment groups were 130.73±14.82 mEq/L and 131.16±9.81 mEq/L respectively (Figure 3.20a), a little lower than the reference range in rats which is 135.7–143.9 mEq/L (Minematsu *et al.*, 1995).

Chloride ion concentration of control and treatment groups were 101.79±1.51 mEq/L and 101.03±6.82 mEq/L respectively (Figure 3.20b), well within the reference range in rats which is 95–106 mEq/L (Minematsu *et al.*, 1995).

Potassium ion concentration of control and treatment groups were 5.24±1.09 mEq/L and 5.12±0.82 mEq/L respectively (Figure 3.20c), well within the reference range in rats which is 4.61–5.57 mEq/L (Minematsu *et al.*, 1995).

Calcium ion concentration of control and treatment groups were 10.85 ± 2.64 mg/dL and 10.17 ± 1.22 mg/dL respectively (Figure 3.21a), the control value was a little higher than the reference range in rats which is 9.04 – 10.58 mg/dL (Minematsu *et al.*, 1995).

Magnesium ion concentration of control and treatment groups were 4.14±1.57 mg/dL and 3.36±0.52 mg/dL respectively (Figure 3.21b), well within the reference range in rats which is 1.60–4.44 mg/dL (Minematsu *et al.*, 1995).

### 3.4.4.3 Urinalysis

***Urine volume***

There were no significant changes ($p<0.05$) in 24-hour total urine volume between the treatment and control groups. The urine volume of rats in the control and treatment groups were 13.84±5.79 ml and 14.25±5.52 ml respectively (Figure 3.22).

***Urinalysis dip-and-read test strips***

The urine of the treatment group did not show any significant difference ($p<0.05$) from the control group in terms of color and the levels of urobilinogen, glucose, ketones, bilirubin, protein, nitrite, pH, blood, specific gravity and leukocytes.

### 3.4.5 Histological study

Photo-micrographs of liver, kidney and thymus sections of both of the control and treated rats were shown in Figures 3.23-3.25. Histological studies showed that the organs of the rats treated with 8% CP solution did not show any observable changes when compared to the control group.

## 3.5 Chronic toxicity test

### 3.5.1 Growth rate

Effect of 6-month and 9-month chronic toxicity treatments were given in Figures 3.26-3.27. All groups of rats were growing steadily. There was no significant difference ($p<0.05$) among the three groups and the final body weights were about 680 g.

### 3.5.2 Food and fluid consumption

For the food and fluid consumption, the three groups had no significant difference ($p<0.05$). The 6-month and 9-month chronic treatments had similar results. For the 6-month treatment, the daily uptake of CP in 0.25% CP-exposed rats was from the beginning of 750 down to 165 mg/kg body weight; while for the 1% CP-exposed rats, the uptake was from the beginning of 3315 to 587 mg/kg body weight a day (Table 3.3).

For the 9-month treatment, the daily uptake of CP in 0.25% CP-exposed rats was from the beginning of 781 down to 144 mg/kg body weight, while the total intake range of CP in 1% CP-exposed rats was from the beginning of 3125 down to 607 mg/kg body weight a day (Table 3.3).

### 3.5.3 Organ-weight

Relative organ weights of thymus, kidneys and liver in the three groups of the 6-month and 9-month chronic treatments were shown in Figure 3.28. Relative organ weights of heart, spleen, brain and testes were given in Figure 3.29. In addition, relative caecal contents were shown in Figure 3.30. All results had no significant difference ($p<0.05$).

### 3.5.4 Clinical pathology tests

#### 3.5.4.1 Haematology

***Red blood cell count***

The red blood cell count of the rats, ranged from 5.49 to 6.94 x $10^6$/$mm^3$, were a little lower than the reference range of 6.7–9.0 x $10^6$/$mm^3$ (Gad and Chengelis, 1992) (Figure 3.31a). No significant difference ($p<0.05$) was found among the groups.

***Haematocrit***

The haematocrit of the rats, ranged from 47.67 to 51.44 %, were within the reference range of 39-55 % (Gad and Chengelis, 1992) (Figure 3.31b). No significant difference ($p<0.05$) was found among the groups.

***Haemoglobin***

The haemoglobin of the rats was ranged from 18.49 to 19.67 g/dL (Figure 3.32a). No significant difference ($p<0.05$) was found among the groups.

***Mean corpuscular volume (MCV)***

The MCV of the rats was ranged from 85.43 to 87.80 $\mu m^3$ (Figure 3.32b). No significant difference ($p<0.05$) was found among the groups.

***Mean corpuscular haemoglobin (MCH)***

Like MCV, there were no significant treatment-related ($p<0.05$) changes observed in MCH among the three groups (Figure 3.32c). The value of MCH of the rats was between 31.69 to 35.48 pg.

***Mean corpuscular haemoglobin concentration (MCHC)***

Effect of 9-month chronic treatment of CP on the MCHC of rats was shown in Figure 3.32d. No significant difference ($p<0.05$) was found among the groups and the MCHC of the rats was ranged from 37.33 to 38.77 %.

***Total white blood cell count***

The reference range of total white blood cell count is 3.0–14.5 x $10^3/mm^3$ (Gad and Chengelis, 1992). In Figure 3.33, the total white-cell count of the rat was ranged from 8.48 to 12.10 x $10^3/mm^3$, which fell within the reference range and with no significant difference ($p<0.05$).

***Differential white blood cell count***

The composition of five types of white blood cells, namely neutrophils, lymphocytes, monocytes, eosinophils and basophils of 6-month and 9-month chronic treatments were shown in Figure 3.34. All groups had similar results. Lymphocytes (ranged from 62.13 to 71.72 %) and neutrophils (ranged from 34.12 to 40.53 %) occupied the majority of white blood cells, while monocytes (ranged from 2.25 to 3.50 %) and eosinophils (ranged from 2.25 to 4.00 %) were in little amount. Basophils were not detected in all the groups.

All of the above cell types fell within the following reference ranges. Neutrophils is 11.5–41.6 %, basophils is 0 %, lymphocytes is 43.0–79.5 %, monocytes is 0–4 % and eosinophils is 0–4 % (Gad and Chengelis, 1992).

### 3.5.4.2 Blood chemistry

**Glucose and lipids**

Effect of chronic treatment of CP on serum glucose (ranged from 92.12-135.74 mg/dL), cholesterol (ranged from 72.06-108.04 mg/dL), triglyceride (ranged from 66.78-100.98 mg/dL), HDL cholesterol (ranged from 50.63-65.47 mg/dL) and LDL cholesterol (ranged from 12.46-22.31 mg/dL) levels were shown in Figures 3.35–3.37. There were no significant treatment-related changes ($p<0.05$) among these parameters.

**Proteins**

Effect of chronic treatment of CP on serum total protein (ranged from 5.64-7.98 g/dL), albumin (ranged from 0.78-1.32 g/dL), globulin (ranged from 4.83-5.48 g/dL), albumin/globulin ratio (0.16-0.26) and immunoglobulins (IgG: ranged from 73.68-100 % of the controls; IgA: ranged from 79.55-100 % of the controls; IgM: ranged from 83.91-100 % of the controls) levels were given in Figures 3.38–3.39. No significant difference ($p<0.05$) was found among the groups except in the serum albumin/globulin ratio of the 6-month chronic treatment. The 1% treatment group ($0.16\pm0.03$) had a significantly ($p<0.05$) lower ratio than that of the control group ($0.20\pm0.04$). However, there was no significant difference at $p<0.01$.

**Liver function**

Serum aspartate transferase (AST) (ranged from 62.53-91.12 U/L), alanine transferase (ALT) (ranged from 18.77-29.86 U/L), alkaline phosphatase (ALP) (ranged from 50.91-69.94 U/L), γ-glutamyl transpeptidase (GGTP) (ranged from 0.44-0.87 Units/mL) and total bilirubin (ranged from 0.18-0.30 mg/dL) levels of the

control and treatment groups were shown in Figures 3.40–3.42. No significant difference ($p<0.05$) was found among the groups.

**Renal function**

Serum urea nitrogen (ranged from 11.78-13.49 mg/dL) and creatinine (ranged from 0.64-0.83 mg/dL) levels; urine creatinine level (ranged from 87.66-151.17 mg/dL) and creatinine clearance (ranged from 0.78-0.83 ml/min) of the rats were given in Figures 3.43–3.44. There were no significant treatment-related changes ($p<0.05$) among the groups.

**Electrolytes**

Levels of serum sodium (ranged from 126.85-139.45 mEq/L), chloride (ranged from 96.88-107.50 mEq/L), potassium (ranged from 5.31-5.86 mEq/L), calcium (ranged from 7.71-9.24 mg/dL) and magnesium (ranged from 2.19-2.66 mg/dL) ions were shown in Figures 3.45-3.46. No significant difference ($p<0.05$) was found.

### 3.5.4.3 Urinalysis

***Urine volume***

The 24-hour total urine volume of the rats was ranged from 8.25-14.00 ml (Figure 3.47). No significant difference ($p<0.05$) was found in the 9-month chronic treatment. However, in the 6-month treatment, the urine volume in the 0.25% treatment group (14.00±4.20 ml) was significantly greater than that of the control (8.25±3.47 ml) and the 1% treatment (10.31±2.24 ml) groups.

***Urinalysis dip-and-read test strips***

The urine of rats in the treatment groups in both 6-month and 9-month chronic treatments did not show any significant difference ($p<0.05$) from the control group in terms of color and the levels of urobilinogen, glucose, ketones, bilirubin, protein, nitrite, pH, blood, specific gravity and leukocytes.

## 3.5.5 Histological study

Photo-Micrographs of liver, kidney, thymus and testis sections of the control (0%) and treatment groups (0.25% and 1%) of the 9-month chronic treatment were shown in Figures 3.48–3.51. Histological studies showed that the organs of the rats treated with CP solution did not show any observable changes when compared to the controls.

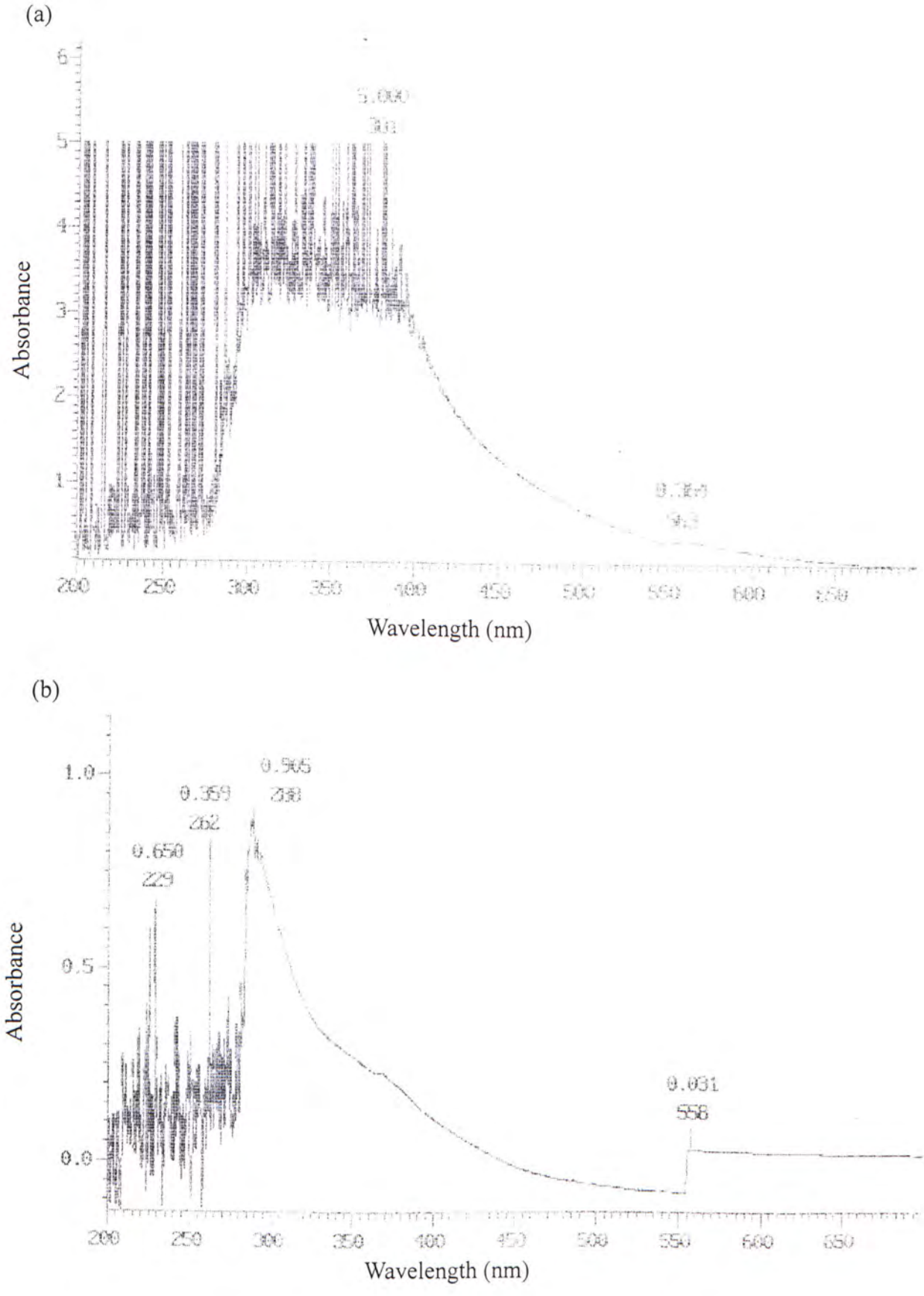



**Figure 3.1 Spectrums of CP.** (a) Purified CP after the purification process of solvent extraction, (b) further purification of CP by reversed-phase HPLC, at retention time of 19 min. The concentration of both CP preparations is 0.1 %.

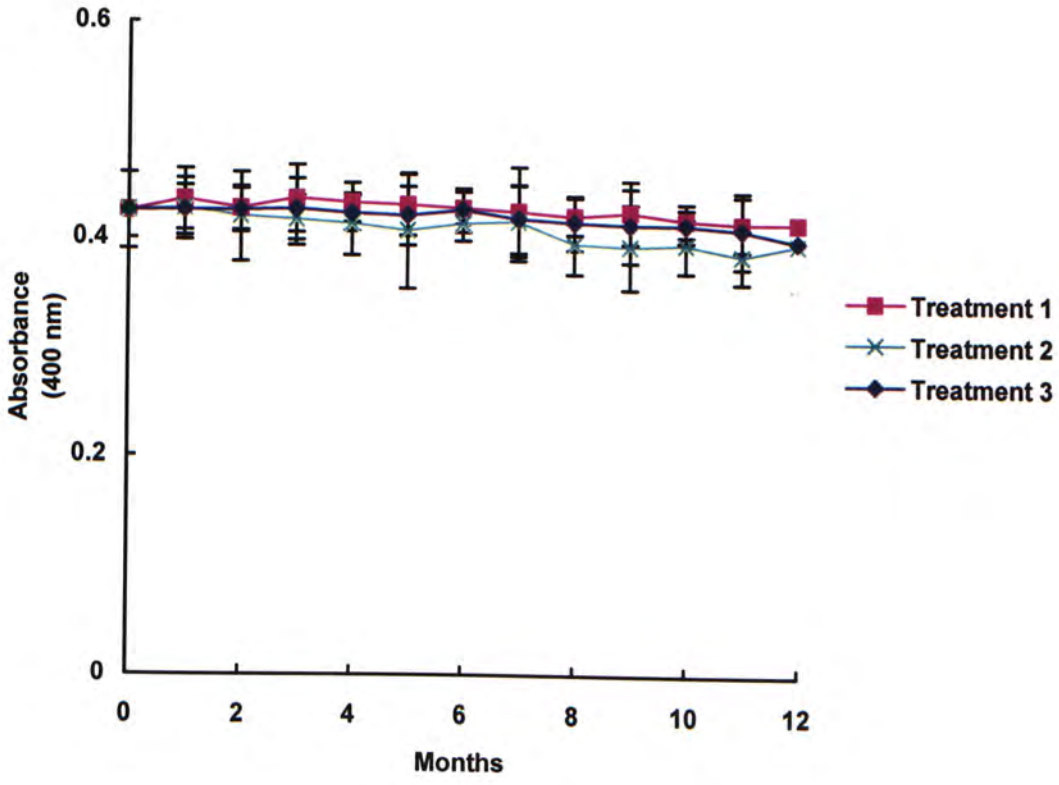



**Figure 3.2 Effects of temperature and light on the stability of CP.** Treatment 1: 0.1% CP stored at room temperature (25 °C) under light; Treatment 2: 0.1% CP stored at room temperature (25 °C) in darkness; Treatment 3: 0.1% CP stored at a 4 °C under light. Results were means ± SD of triplicate measurements. All results had no significant difference ($p<0.05$).

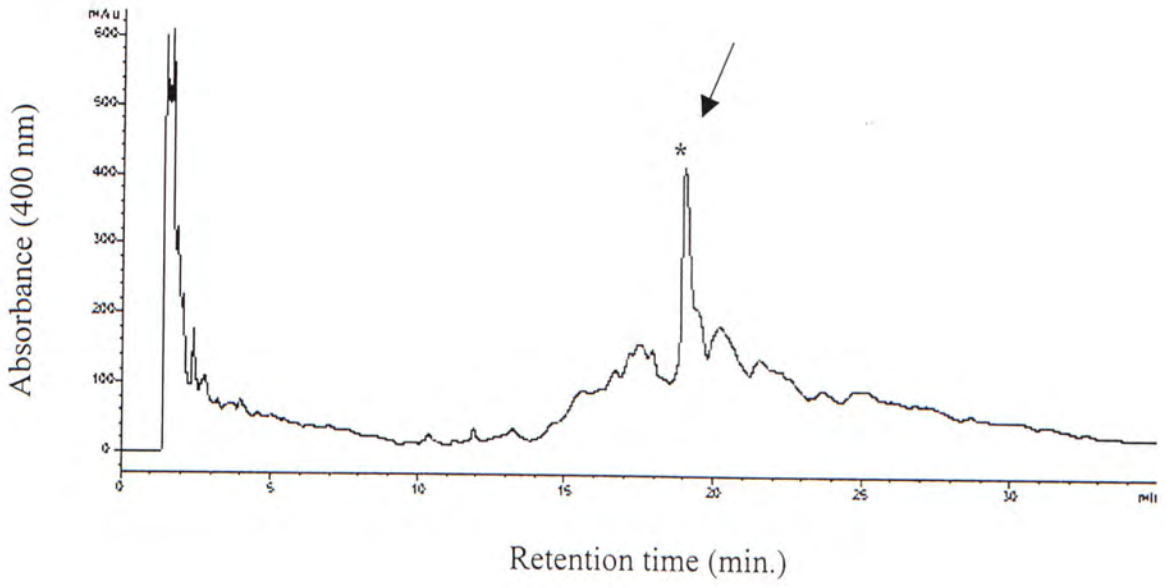



**Figure 3.3 HPLC profile of CP.** * Retention time at 19 min.

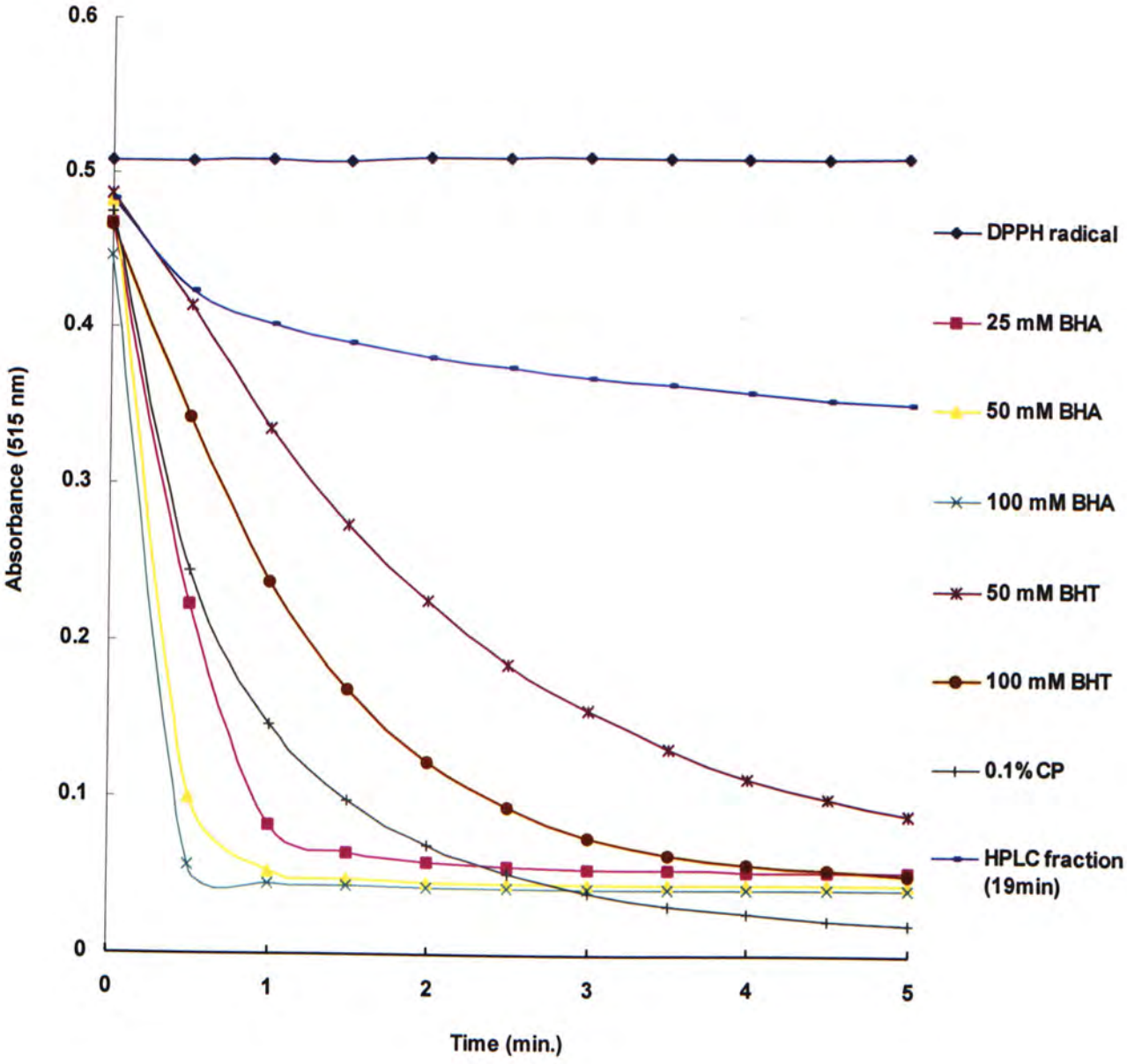



**Figure 3.4 Hydrogen donating abilities of 0.1% CP, HPLC fraction and standards.** The kinetics of samples and standards with DPPH$^{\bullet}$ were plotted. Only one set of representative data was shown.

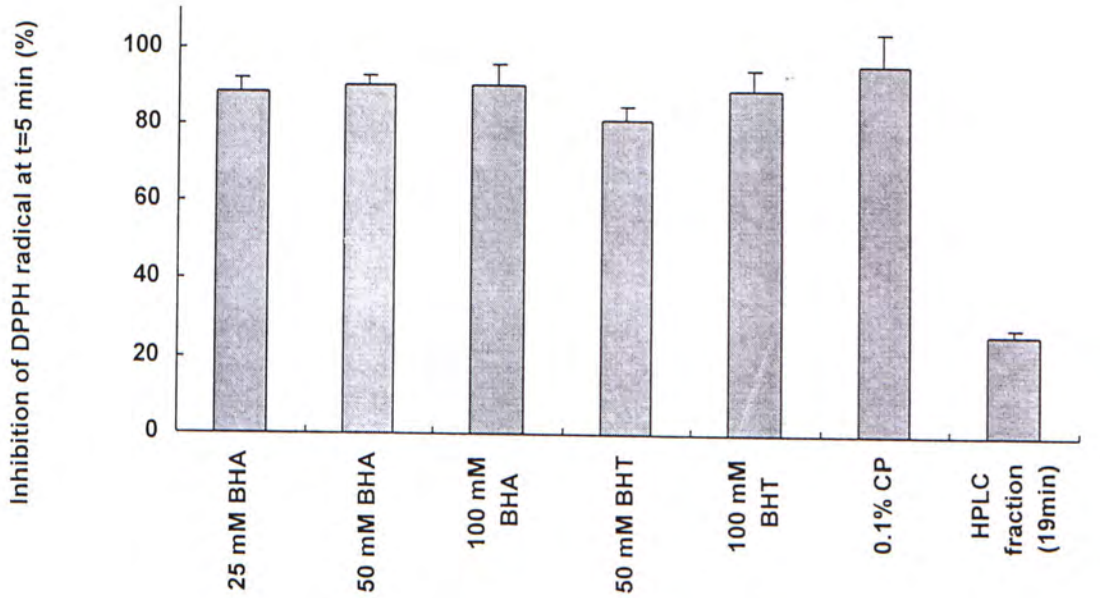



**Figure 3.5 Free radical scavenging abilities of 0.1% CP, HPLC fraction and standards.** Results were means ± SD of triplicate measurements.

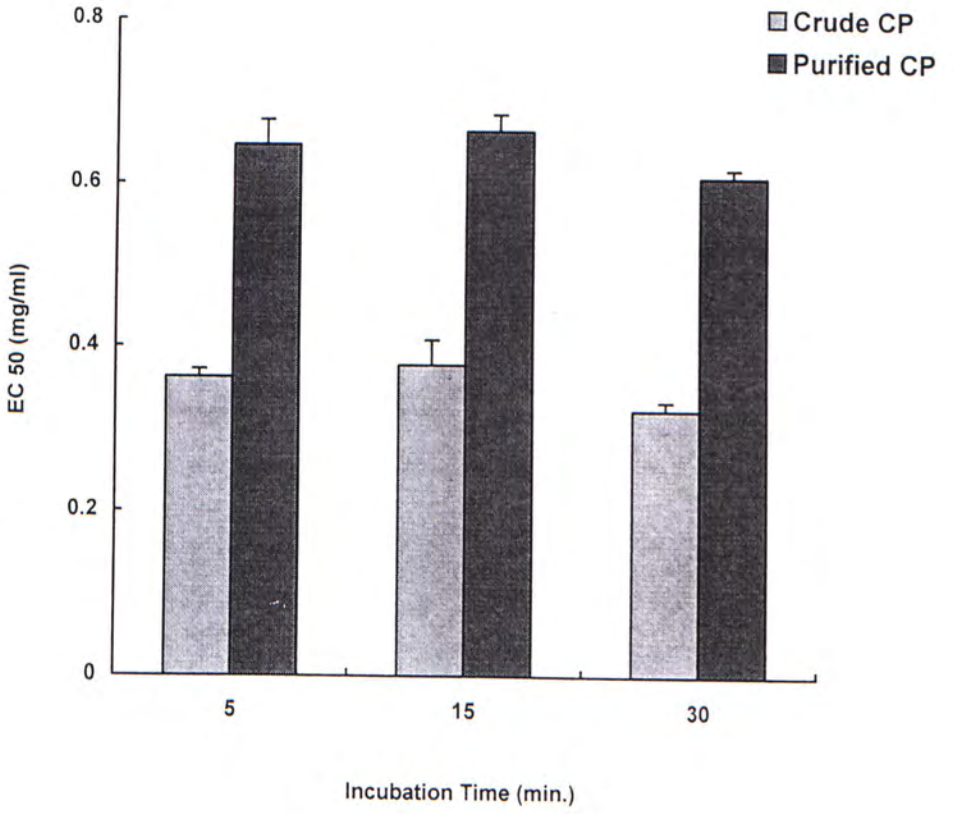



**Figure 3.6 Toxicity of CP in Microtox® test.** Results were means ± SD of triplicate measurements.

**Table 3.1 Mutagenicity of CP in Mutatox® test.**

| | Reading of the Highest Light Level* |
|---|---|
| Media control (mean) | 2 ± 1 |
| Purified CP# | 2 ± 1 (< 4) |

#The concentration of CP used in the test was 0.65 mg/ml which was the EC 50 in Microtox® test.

*Suspected mutagenic agents are defined as those samples which induce or increase light levels to at least two consecutive sample dilution cuvettes.

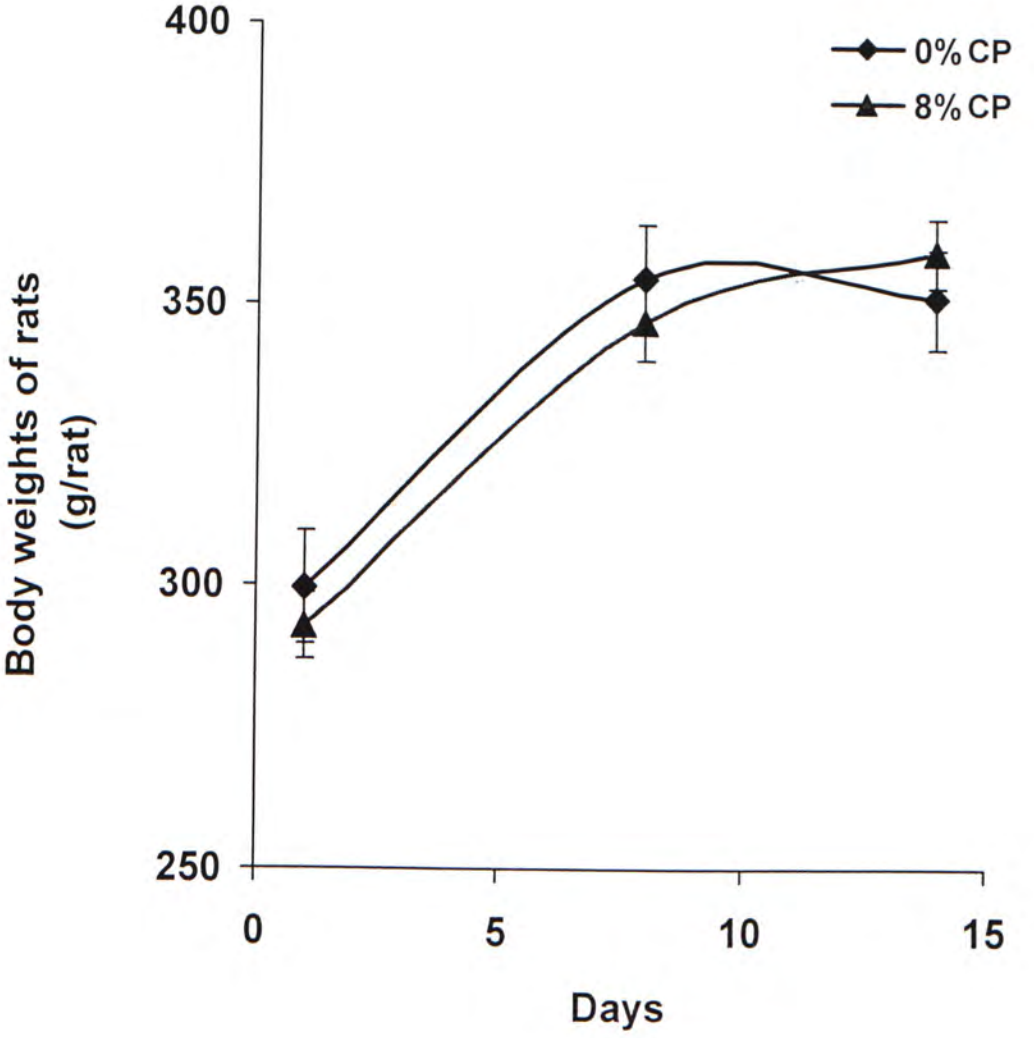


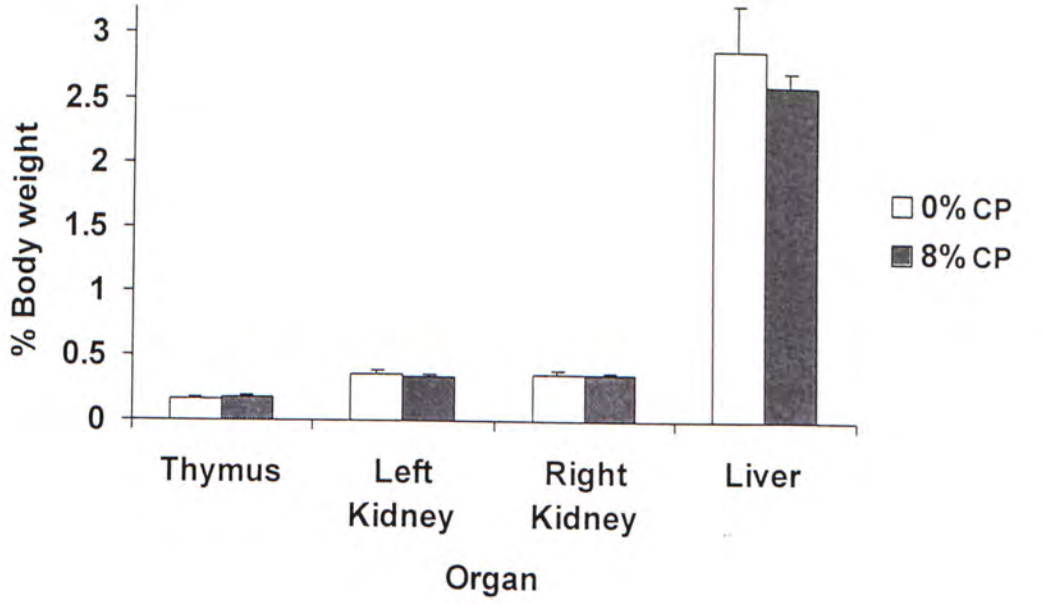

**Figure 3.7 Effect of acute treatment of CP on the body weight of rats.** Concentration of purified CP exposed to rats were 0% and 8%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

**Table 3.2 Consumption of drinking fluid and CP of each group in acute toxicity test.**

| Acute Toxicity Test | | | | |
|---|---|---|---|---|
| Group number | % CP in drinking fluid | Number of rats | Average consumption of drinking fluid per day (ml/day) | Total intake of CP per day (mg/kg body weight) |
| 1 | 0% | 8 | 30.62 ± 4.09 | 0 |
| 2 | 8% | 8 | 32.08 ± 2.97 | 7143 ± 109 |

Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

(a)




(b)

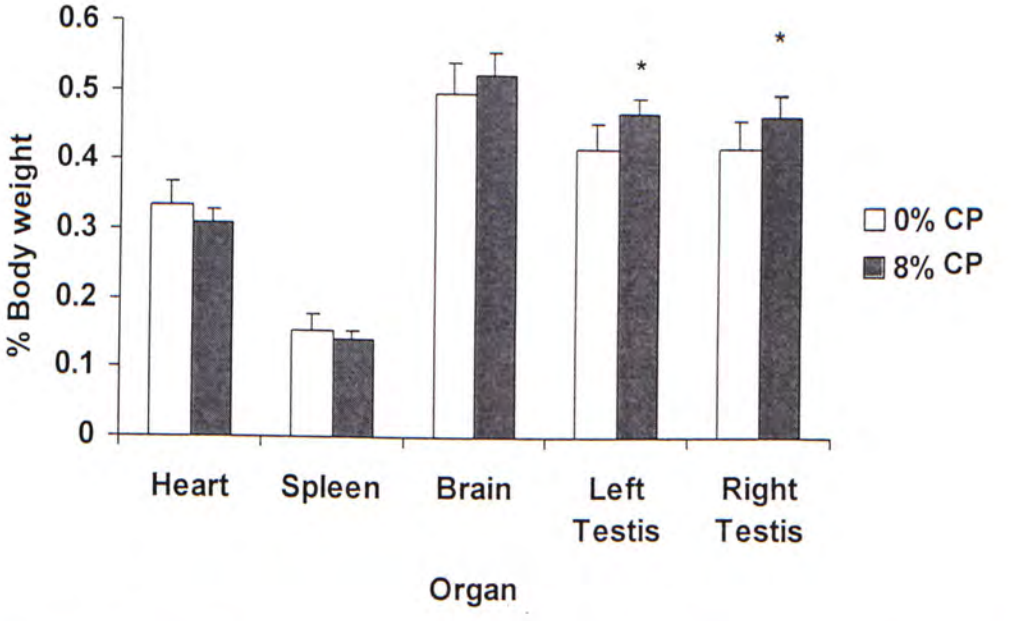



**Figure 3.8 Effect of acute treatment of CP on the relative organ weights of rats.** (a) organs of thymus, kidneys and liver; (b) organs of heart, spleen, brain and testes. Concentration of purified CP exposed to rats were 0% and 8%. Results were means ± SD, no. of rats per treatment = 8. *Results having no significant difference at $p<0.01$. All the other results had no significant difference ($p<0.05$).

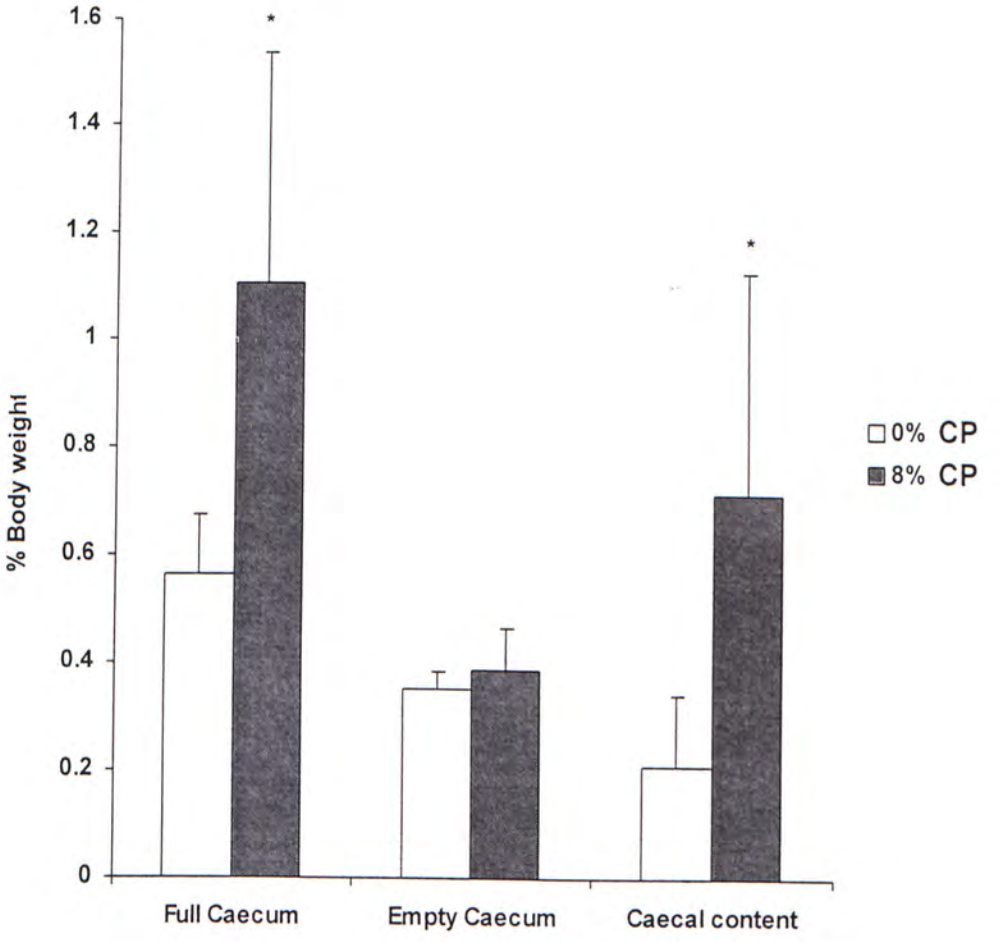
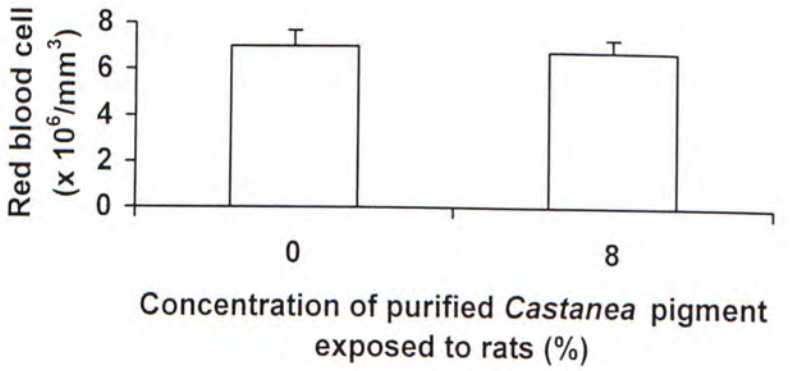
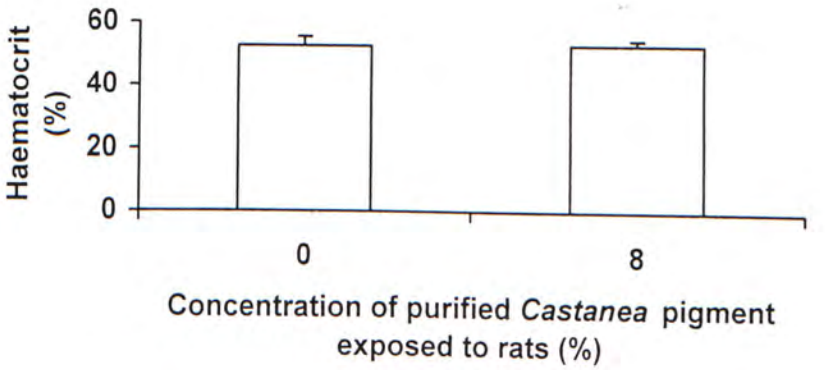



**Figure 3.9 Effect of acute treatment of CP on the relative weight of caecal contents of rats.** Concentration of purified CP exposed to rats were 0% and 8%. Results were means ± SD, no. of rats per treatment = 8. * Significantly difference comparing with the control at $p<0.05$.

(a)




(b)




(c)

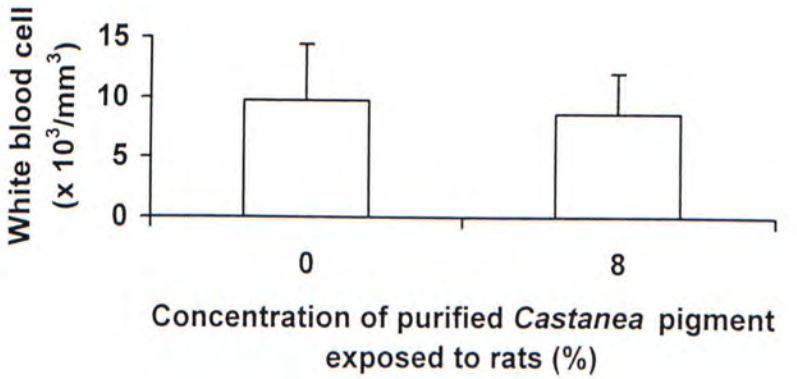



**Figure 3.10 Effect of acute treatment of CP on blood chemistry of rats.** (a) red blood cell counts; (b) haematocrit; (c) total white blood cell counts. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

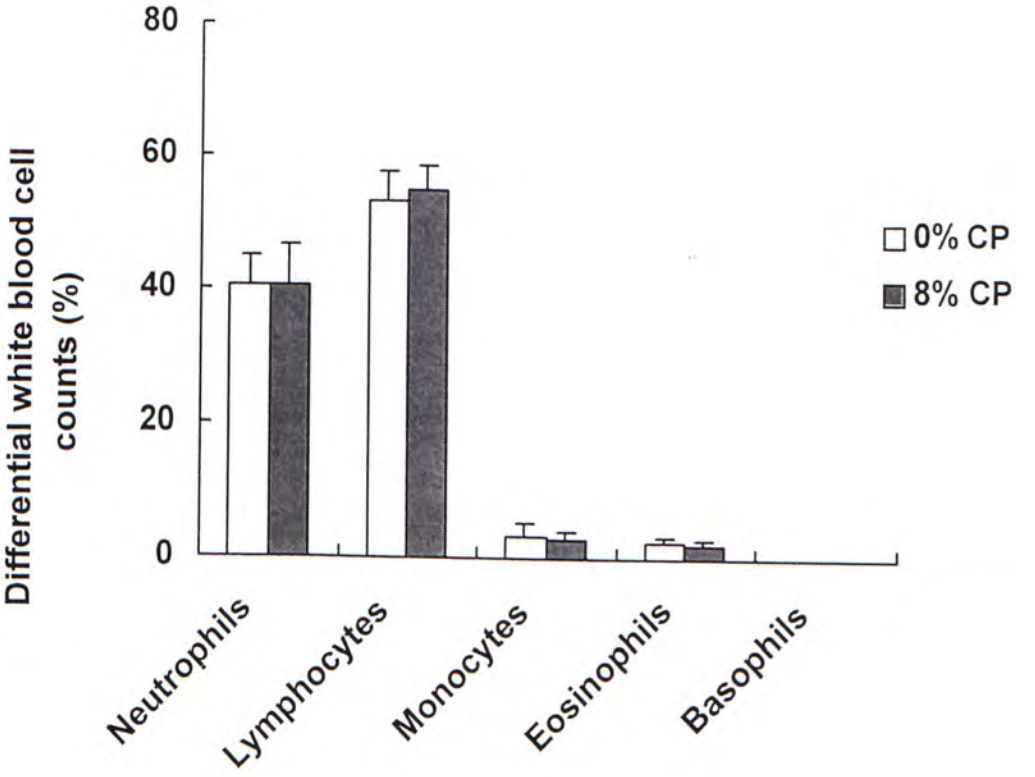



**Figure 3.11 Effect of acute treatment of CP on the differential white blood cell counts of rats.** Concentration of purified CP exposed to rats were 0% and 8%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

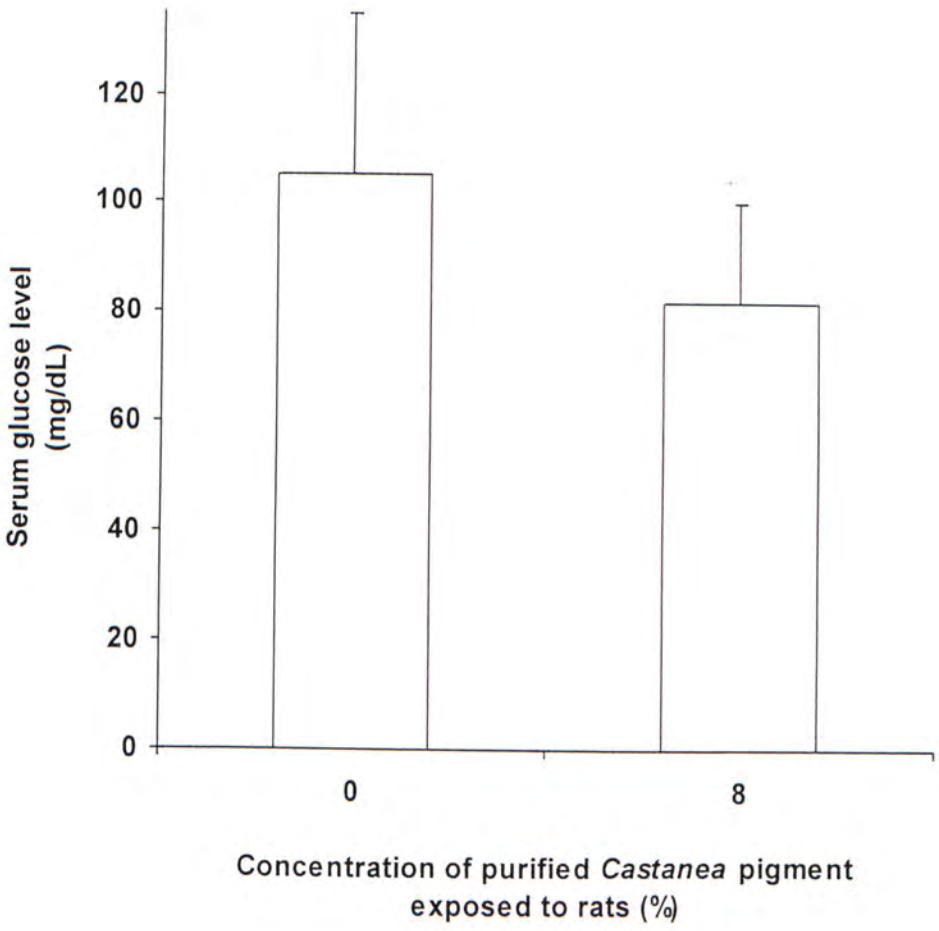



**Figure 3.12 Effect of acute treatment of CP on serum glucose levels of rats.** Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

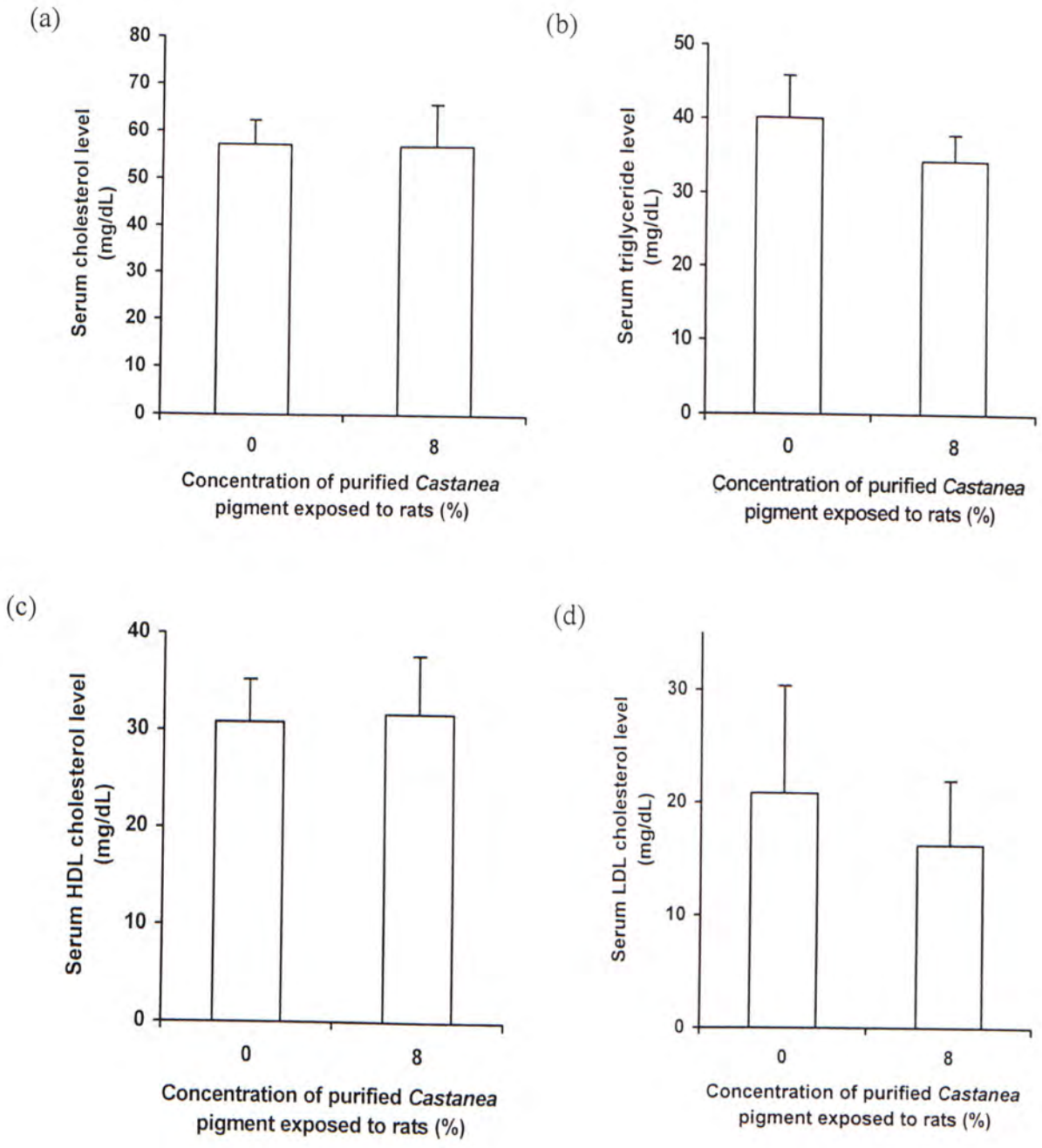



**Figure 3.13 Effect of acute treatment of CP on serum lipids of rats.** (a) cholesterol; (b) triglyceride; (c) HDL cholesterol; (d) LDL cholesterol. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

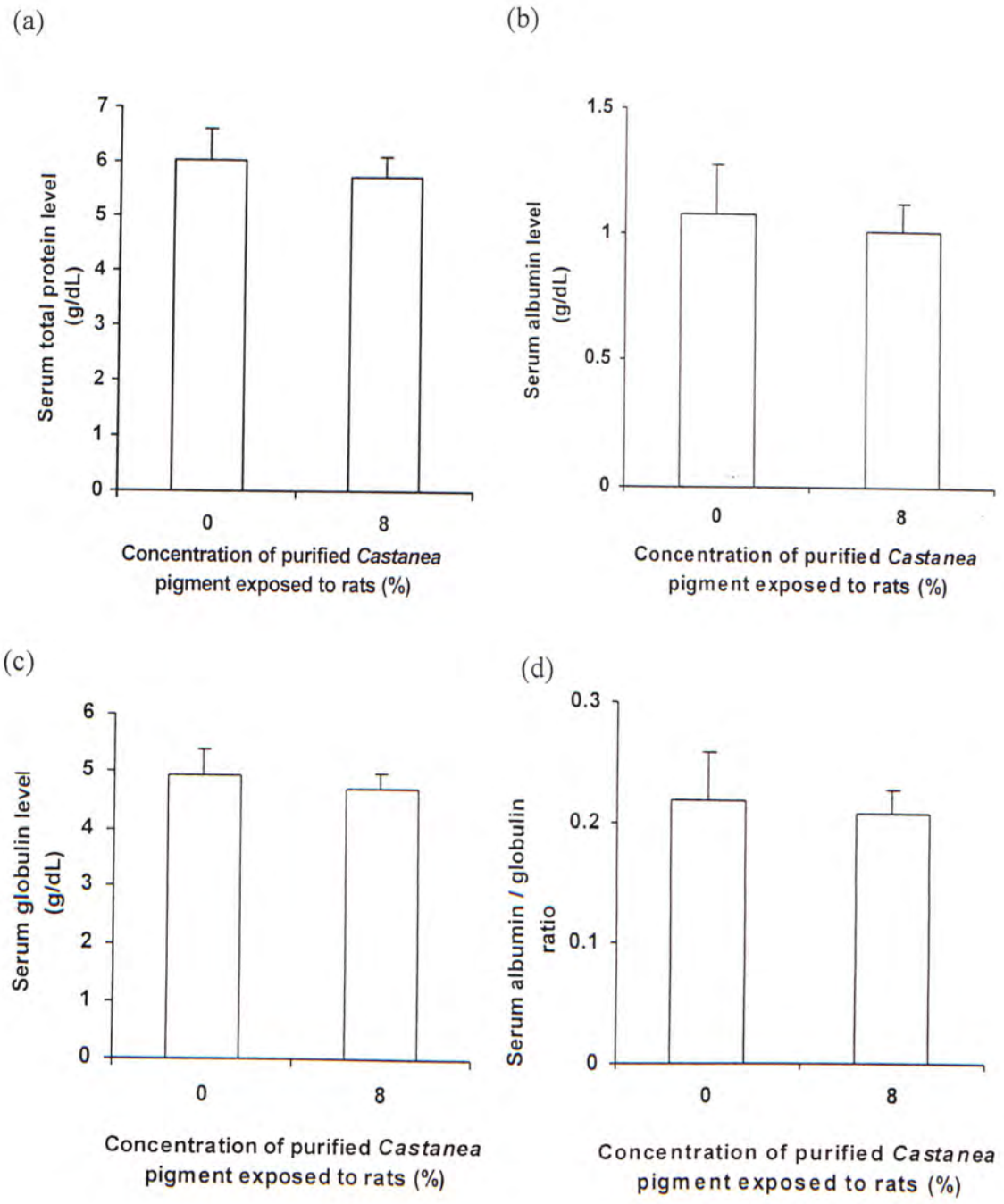
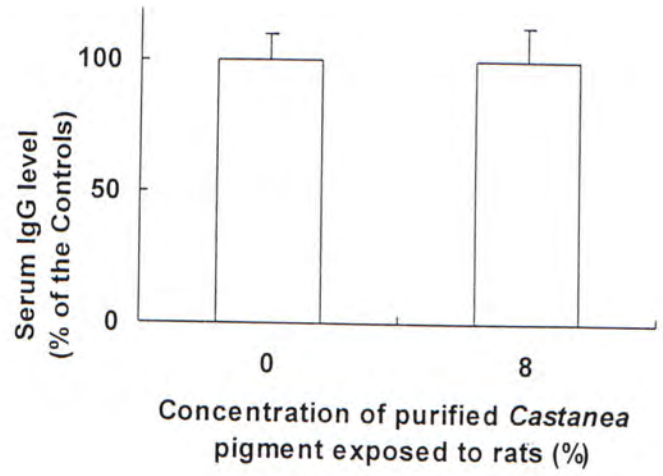
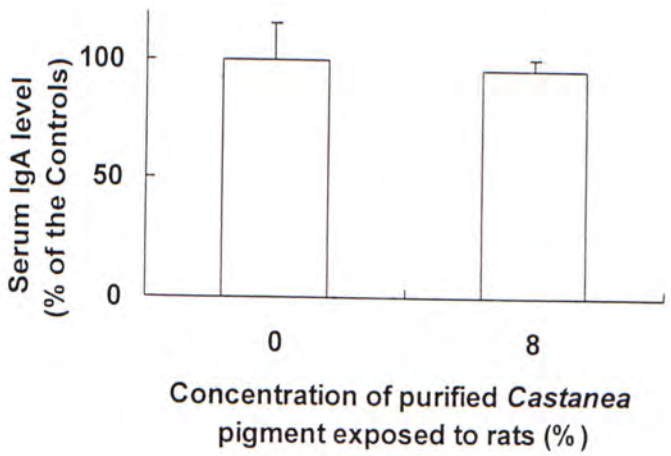



**Figure 3.14 Effect of acute treatment of CP on serum proteins of rats.** (a) total protein; (b) albumin; (c) globulin; (d) albumin/globulin ratio. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

(a)




(b)




(c)

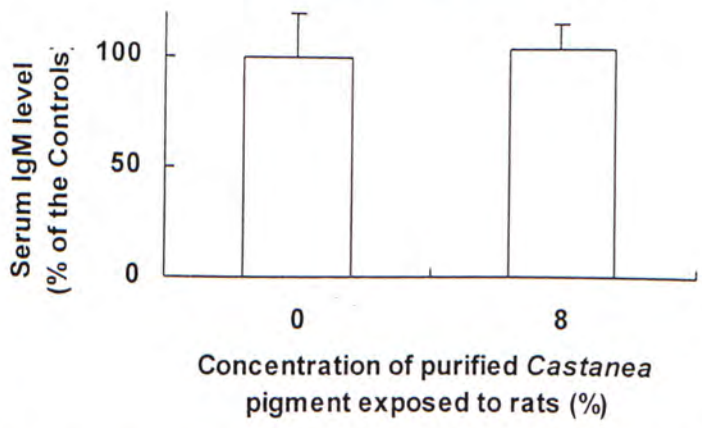



**Figure 3.15 Effect of acute treatment of CP on serum immunoglobulins of rats.** (a) IgG; (b) IgA; (c) IgM. Results were means ± SD, no. of rats per treatment = 8 and were expressed as a percentage of the controls.

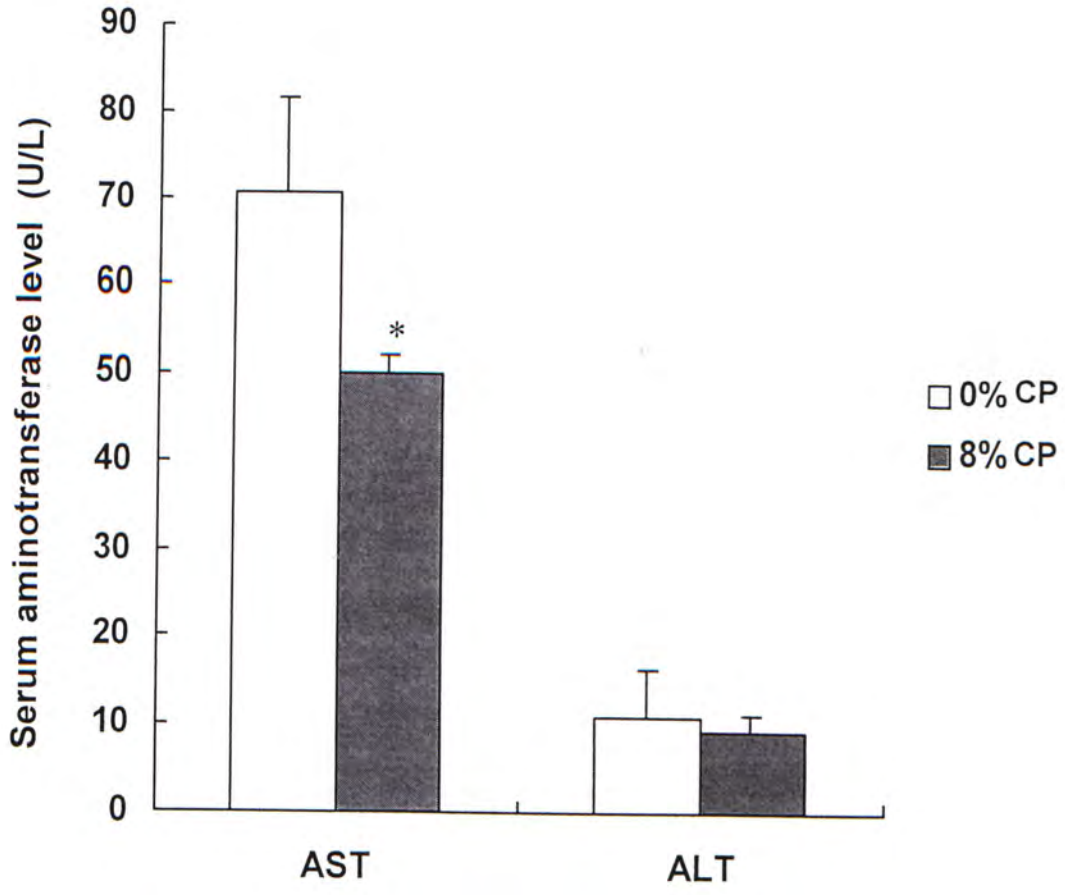
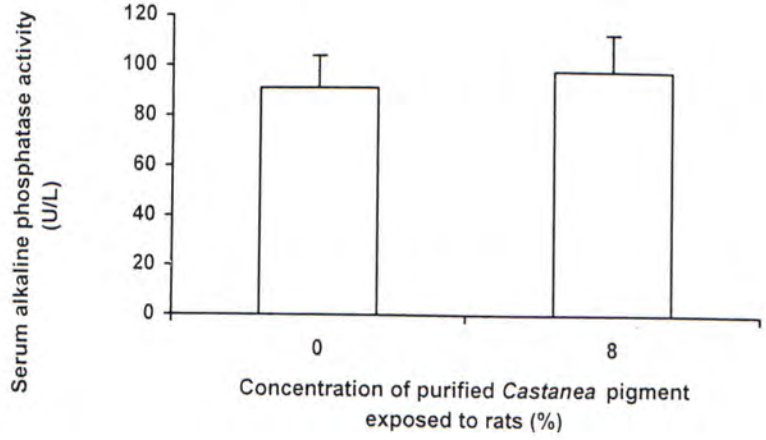
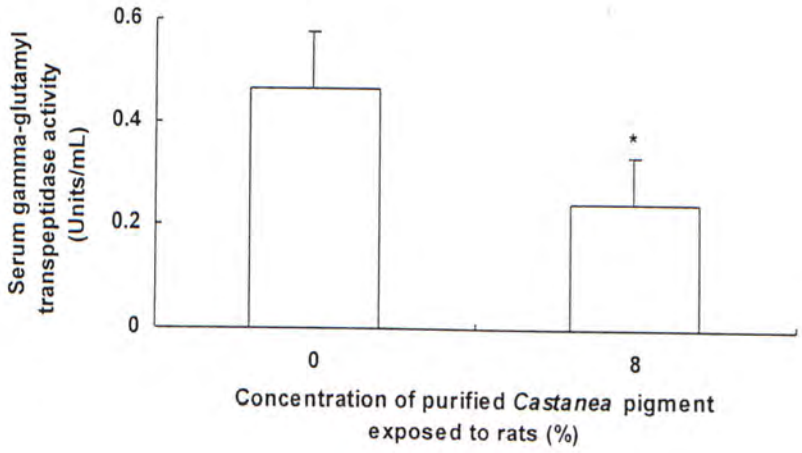



**Figure 3.16 Effect of acute treatment of CP on serum AST and ALT levels of rats.** Concentration of purified CP exposed to rats were 0% and 8%. Results were means ± SD, no. of rats per treatment = 8. * Significantly difference when comparing with the control at $p<0.05$. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase

(a)




(b)

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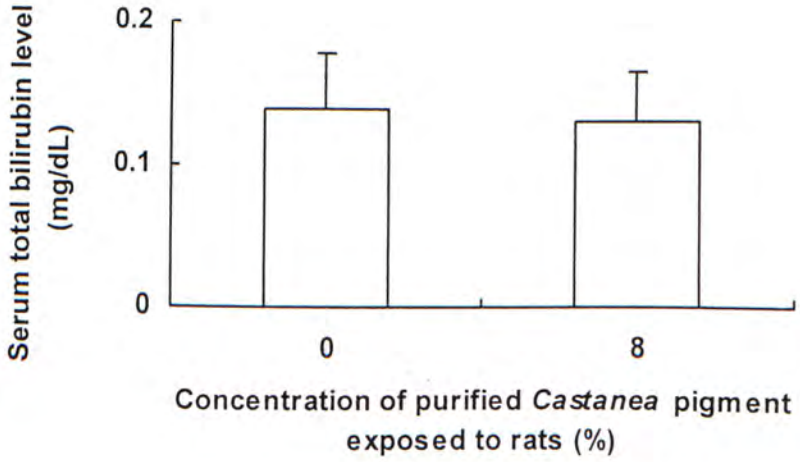



**Figure 3.17 Effect of acute treatment of CP on serum liver enzymes and bilirubin of rats.** (a) alkaline phosphatase; (b) γ-glutamyl transpeptidase; (c) total bilirubin. Results were means ± SD, no. of rats per treatment = 8. * Significantly difference comparing with the control at $p<0.05$. All the other results had no significant difference ($p<0.05$).

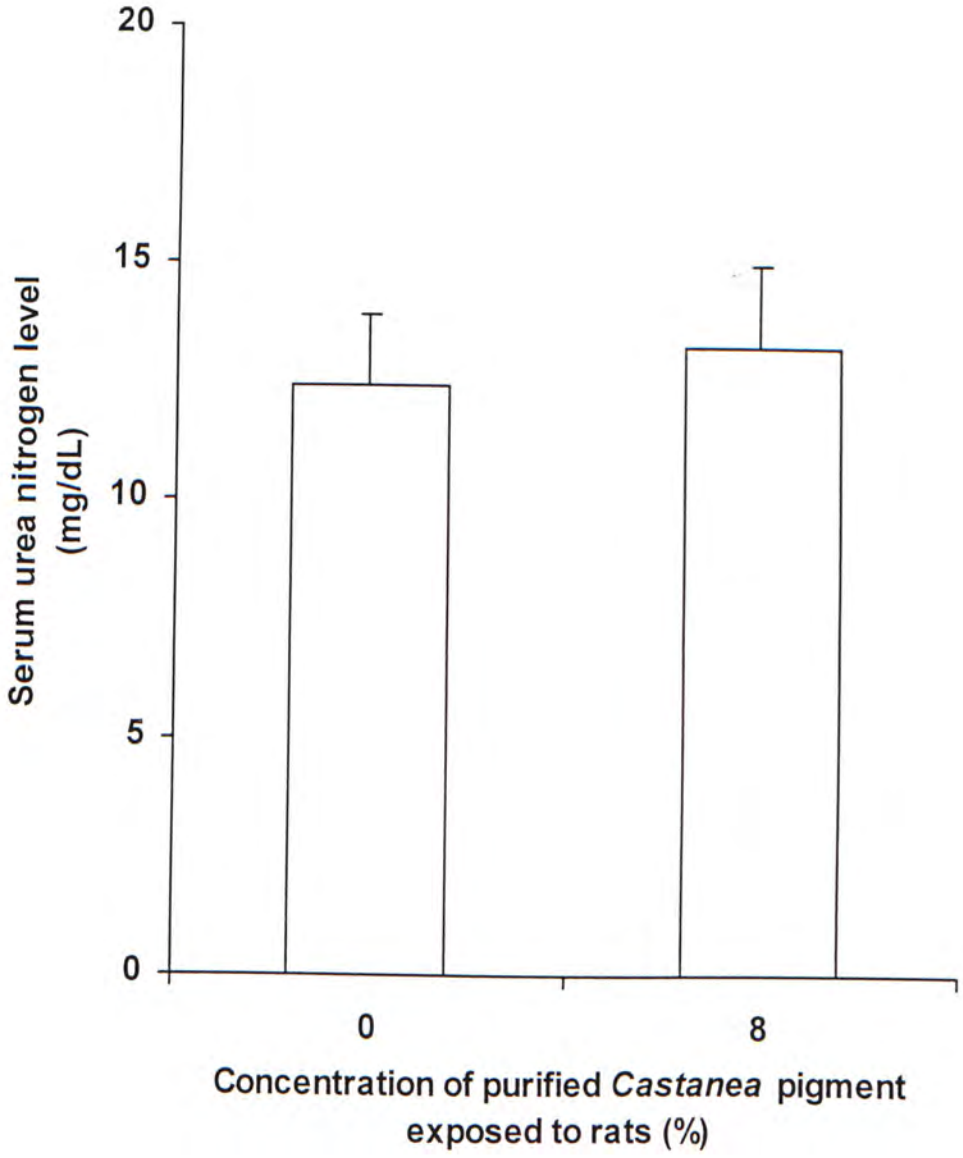
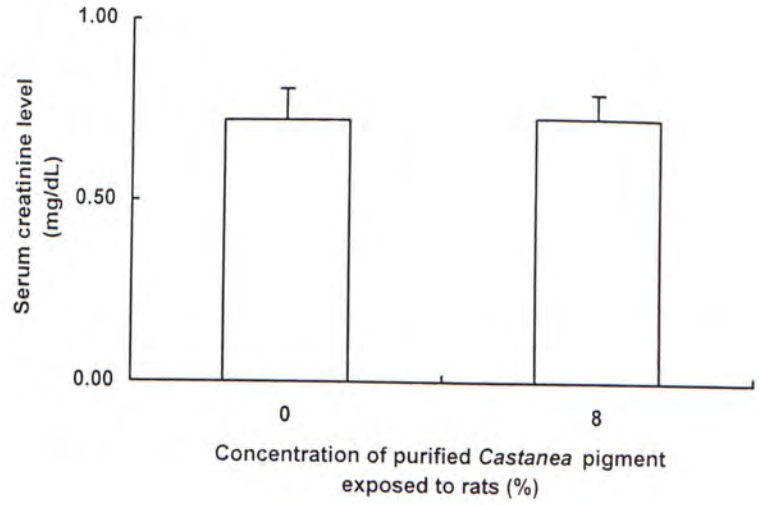



**Figure 3.18 Effect of acute treatment of CP on serum urea nitrogen levels of rats.** Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

(a)

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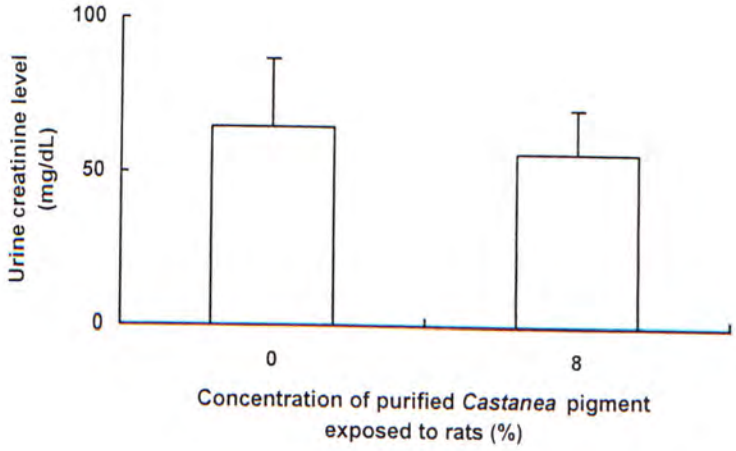


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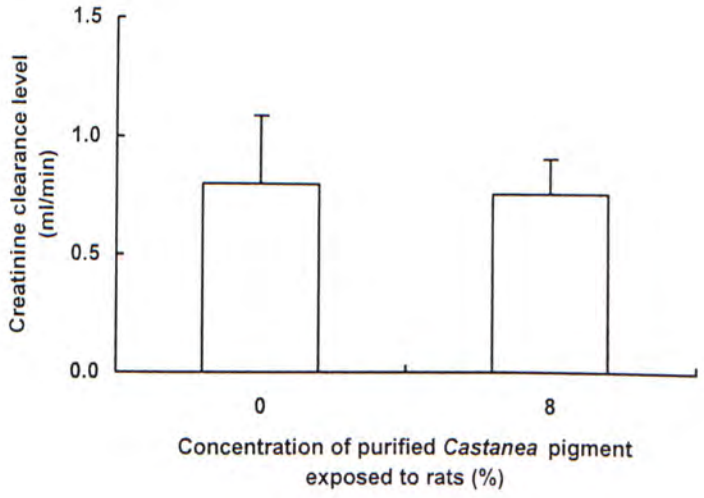
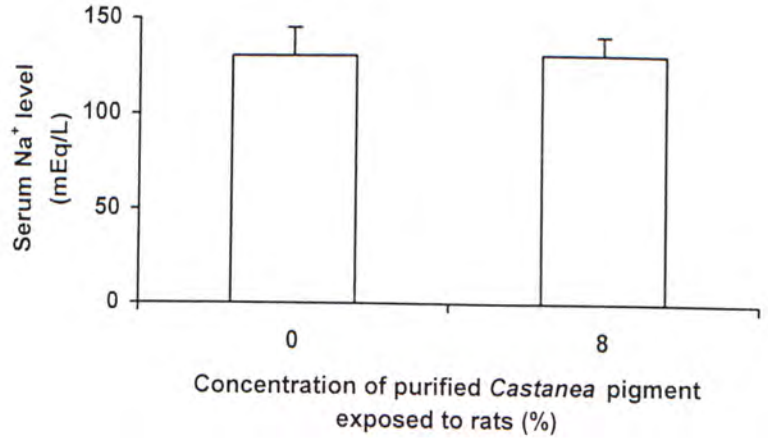
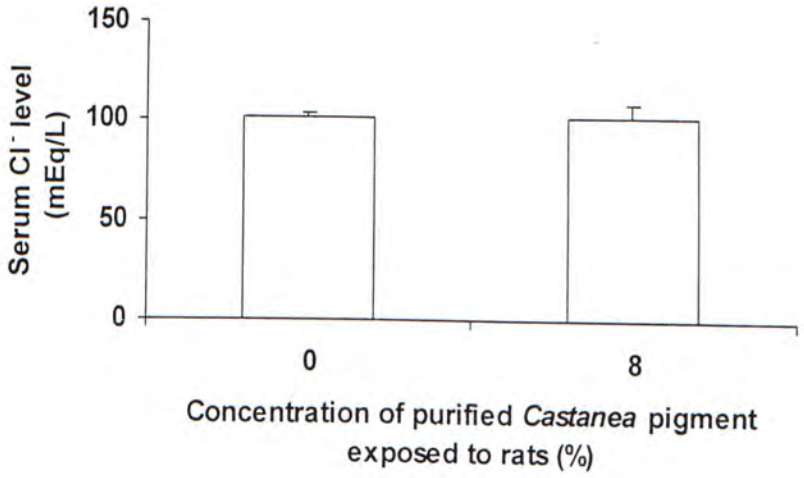



**Figure 3.19 Effect of acute treatment of CP on creatinine of rats.** (a) serum creatinine; (b) urine creatinine; (c) creatinine clearance. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

(a)




(b)




(c)

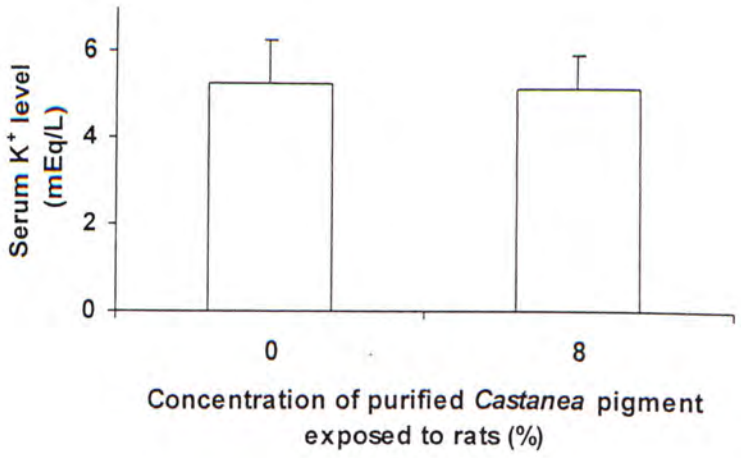
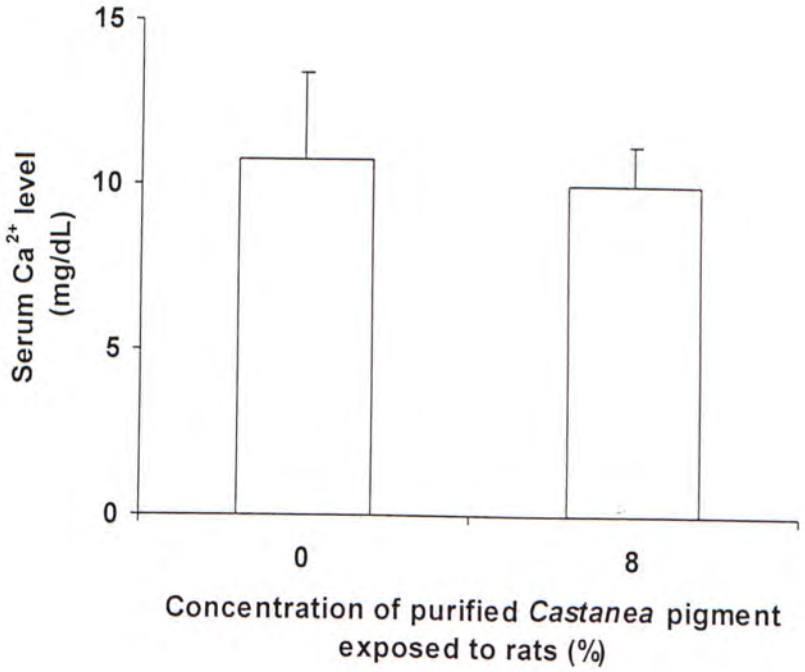



**Figure 3.20 Effect of acute treatment of CP on serum monovalent ions of rats.** (a) sodium ion; (b) chloride ion; (c) potassium ion. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

(a)




(b)

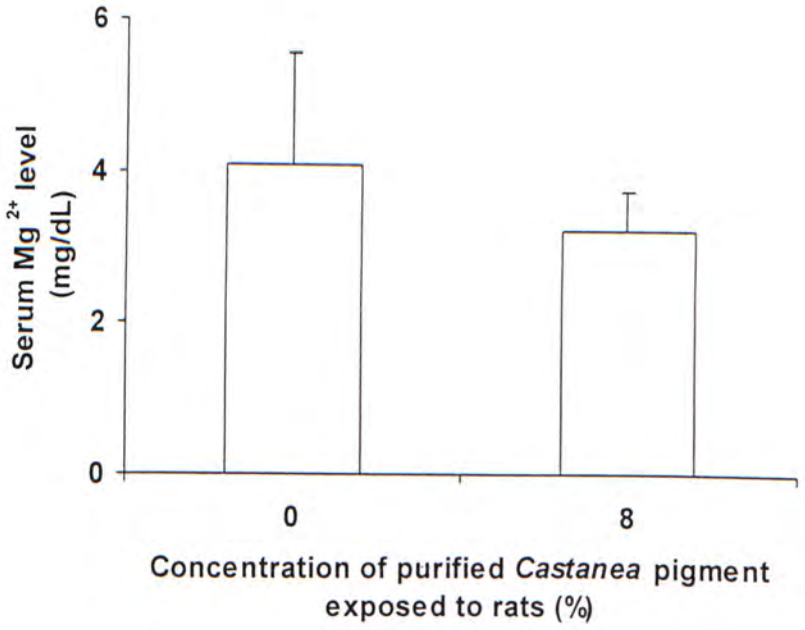



**Figure 3.21 Effect of acute treatment of CP on serum divalent ions of rats.** (a) calcium ion; (b) magnesium ion. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

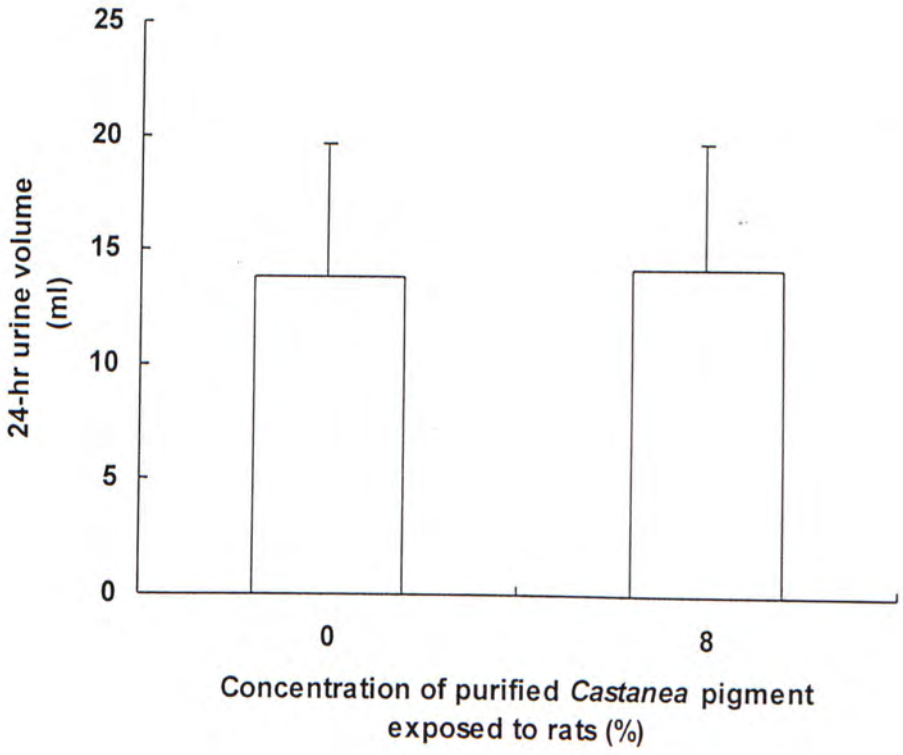



**Figure 3.22 Effect of acute treatment of CP on 24-hr urine volumes of rats.** Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

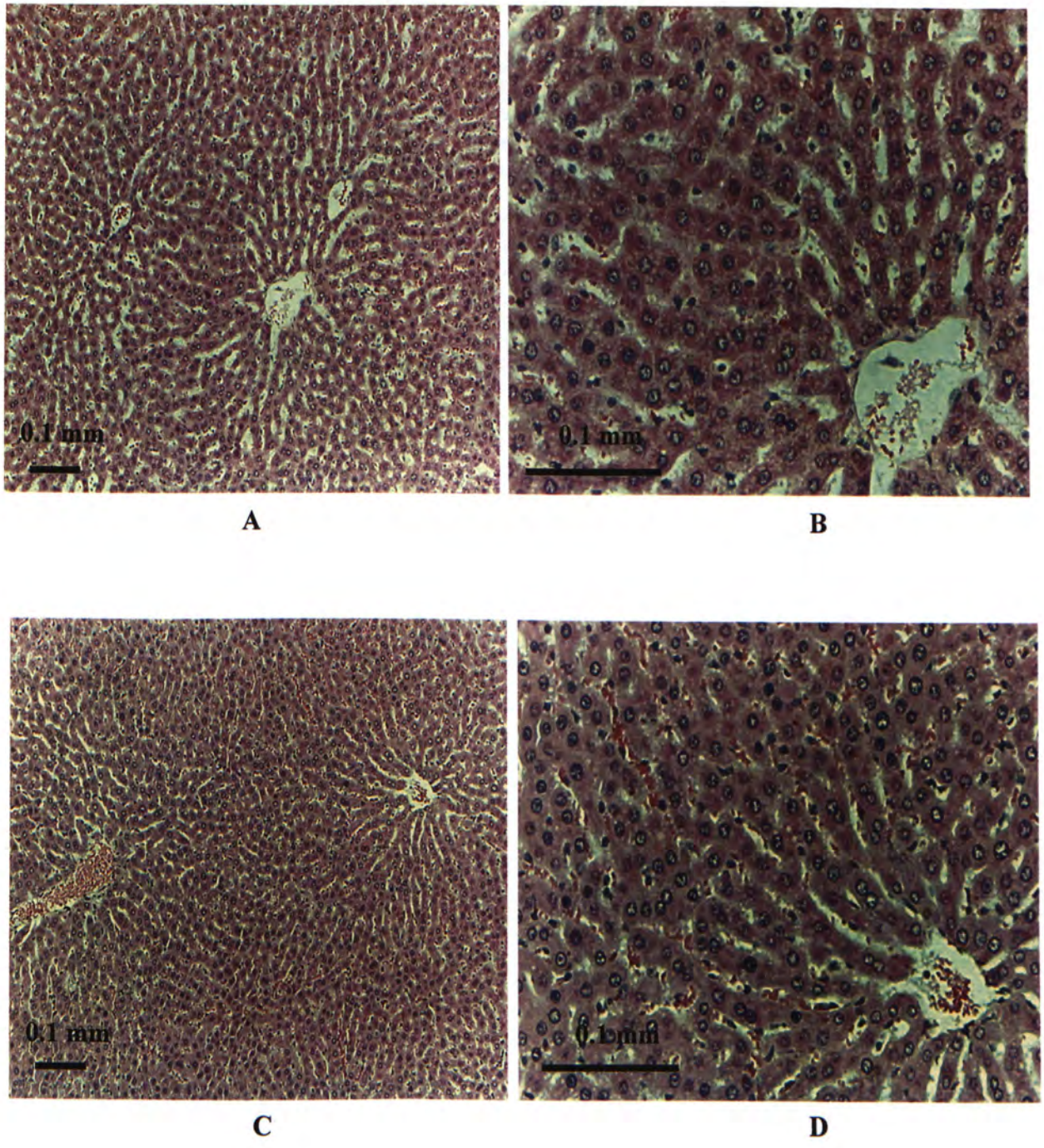
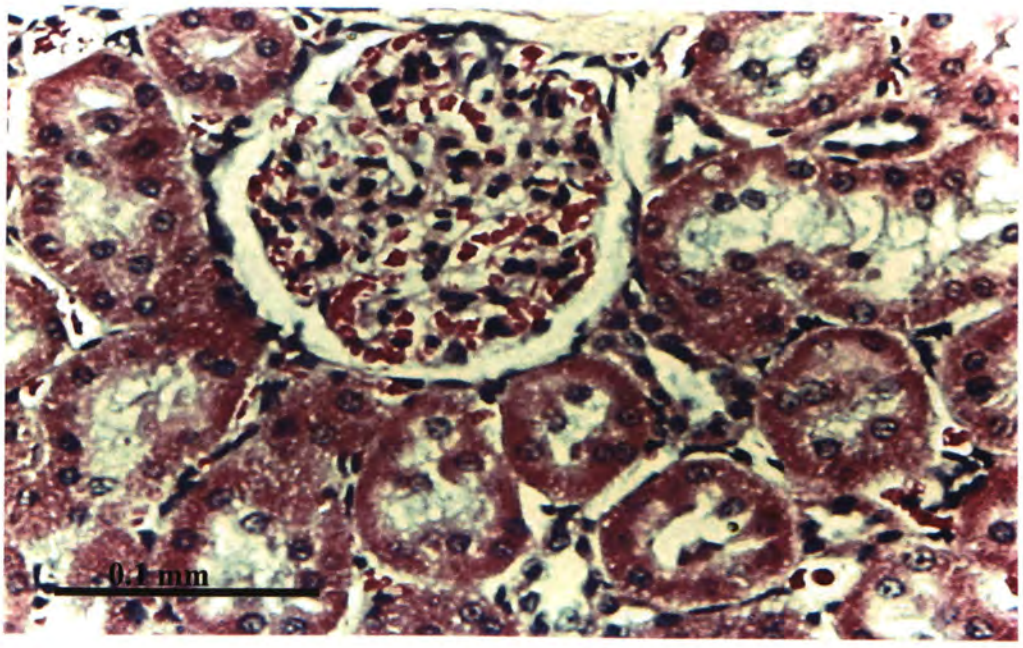
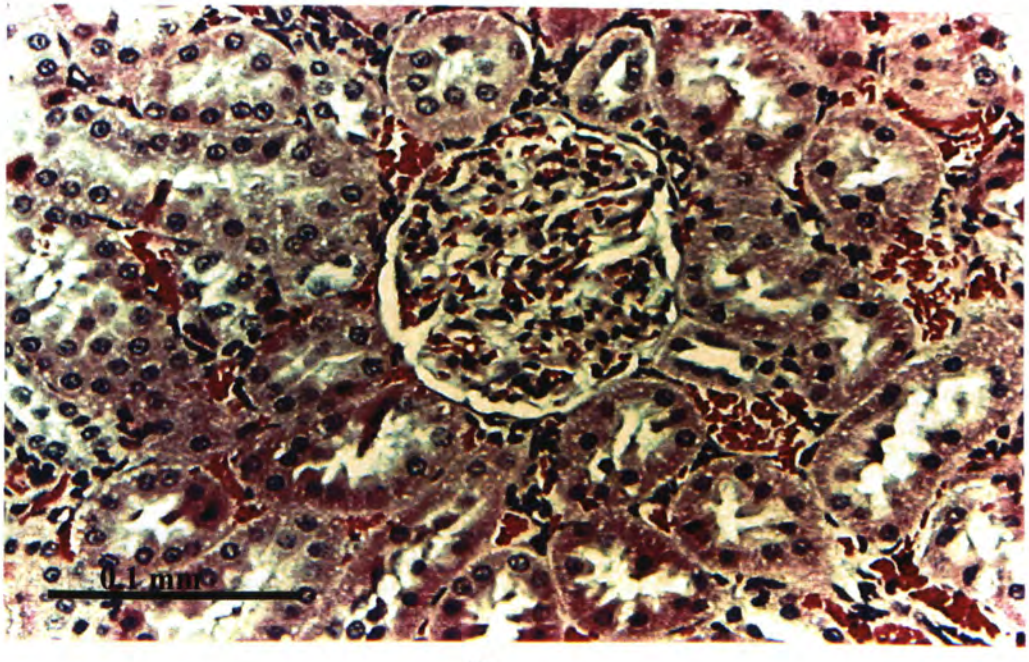



**Figure 3.23 Photomicrographs of liver sections from rats of the acute toxicity test (H&E).** A. & B. Control; C. & D. 8% CP.

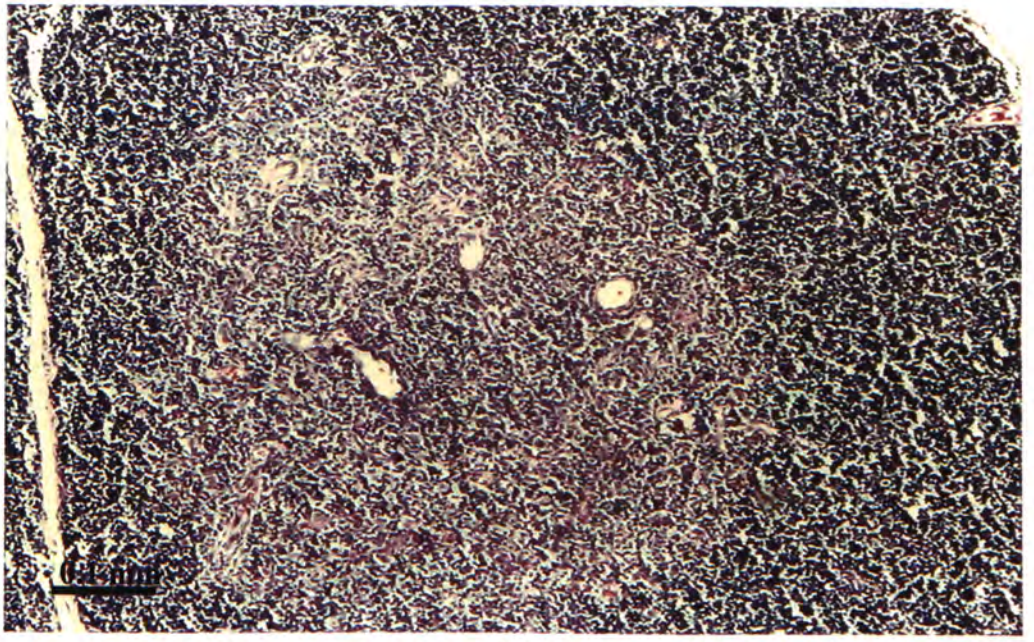



A

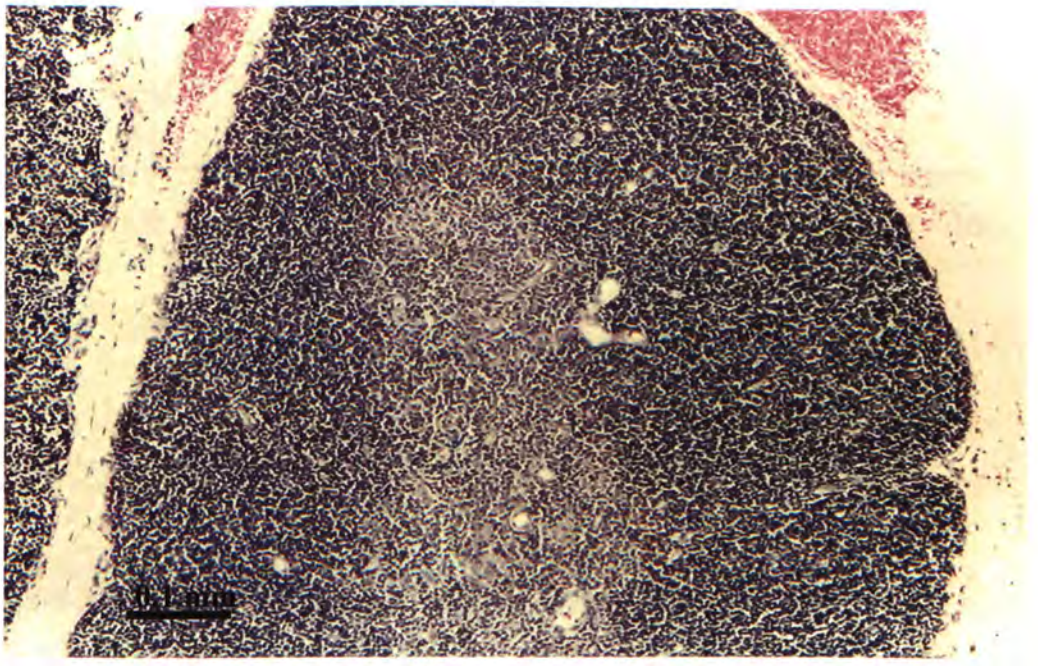



B

**Figure 3.24 Photomicrographs of kidney sections from rats of the acute toxicity test (H&E).** A. Control; B. 8% CP.



A



B

**Figure 3.25 Photomicrographs of thymic sections from rats of the acute toxicity test (H&E).** A. Control; B. 8% CP.

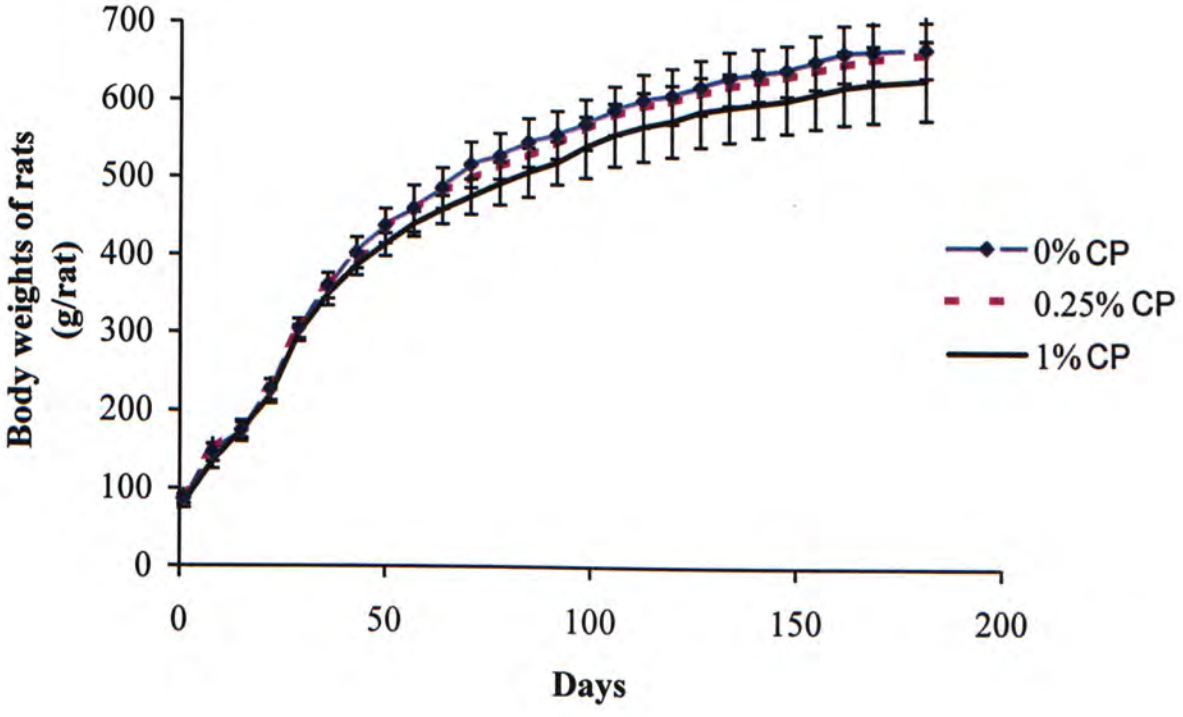



**Figure 3.26 Effect of 6-month chronic treatment of CP on the body weight of rats.** Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

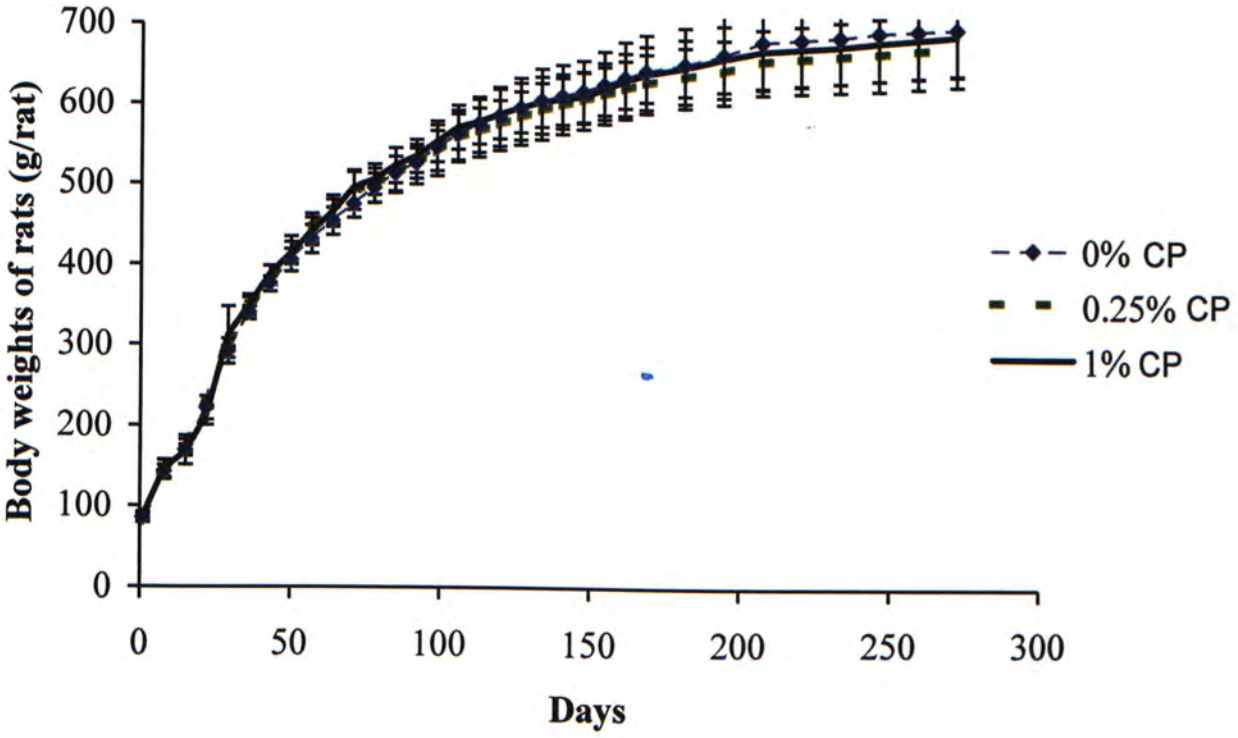



**Figure 3.27 Effect of 9-month chronic treatment of CP on the body weight of rats.** Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

**Table 3.3 Consumption of drinking fluid and CP of each group in 6-month and 9-month chronic toxicity tests.**

| 6-Month Chronic Toxicity Test | | | | |
|---|---|---|---|---|
| Group number | % CP in drinking fluid | Number of rats | Average consumption of drinking fluid per day (ml/day) | Total intake of CP per day (mg/kg body weight) |
| 1 | 0% | 8 | 26.17 ± 1.50 – 40.50 ± 3.67 | 0 |
| 2 | 0.25% | 8 | 24.84 ± 3.14 – 37.34 ± 6.15 | 165 ± 23 – 750 ± 43 |
| 3 | 1% | 8 | 25.07 ± 2.89 – 38.17 ± 5.71 | 587 ± 56 – 3315 ± 133 |
| **9-Month Chronic Toxicity Test** | | | | |
| Group number | % CP in drinking fluid | Number of rats | Average consumption range of drinking fluid per day (ml/day) | Total intake range of CP per day (mg/kg body weight) |
| 1 | 0% | 8 | 26.17 ± 1.50 – 40.50 ± 3.67 | 0 |
| 2 | 0.25% | 8 | 24.84 ± 3.14 – 37.34 ± 6.15 | 144 ± 11 – 781 ± 53 |
| 3 | 1% | 8 | 25.07 ± 2.89 – 38.17 ± 5.71 | 607 ± 85 – 3125 ± 137 |

Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

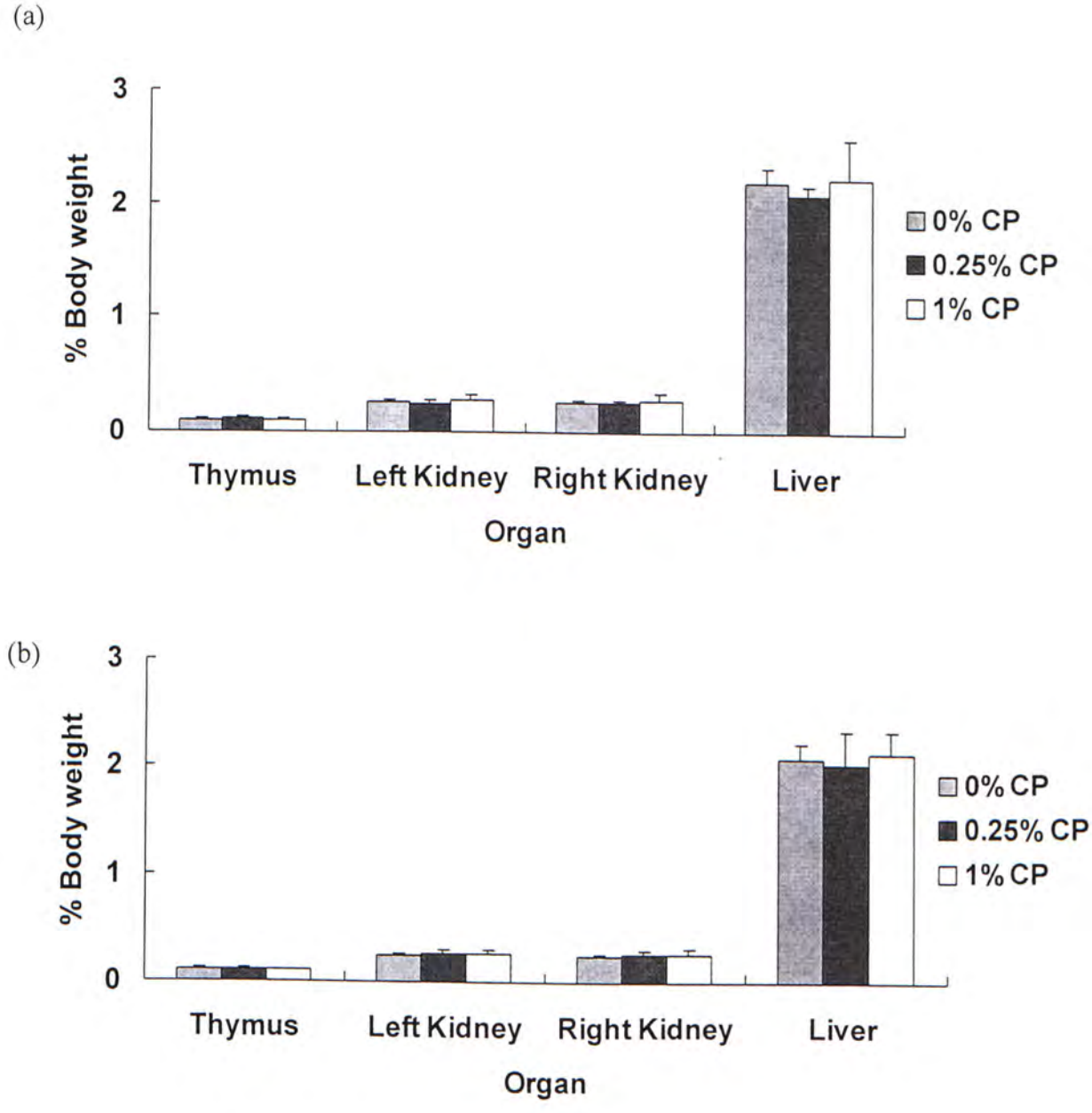
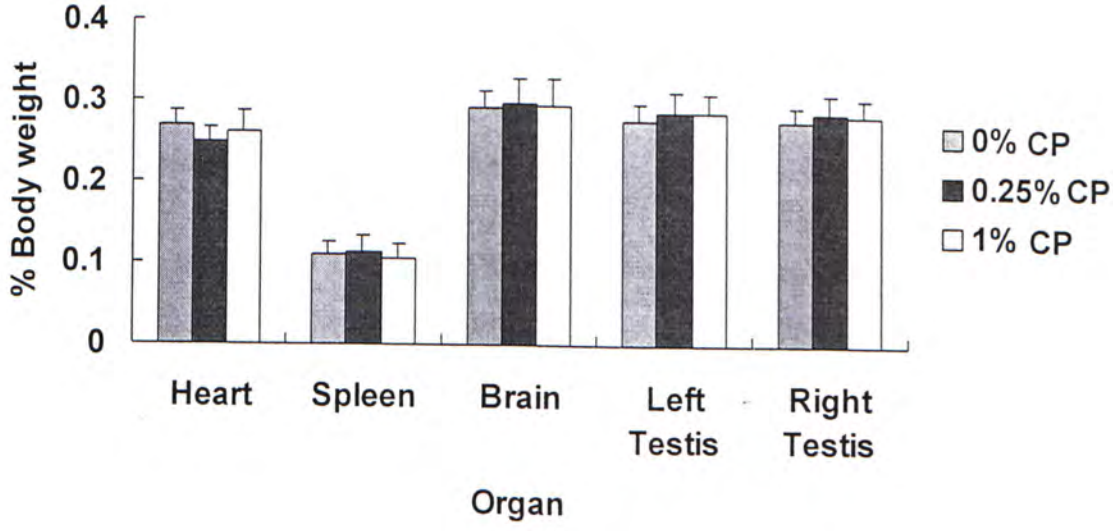



**Figure 3.28 Effect of chronic treatments of CP on the relative organ weights (thymus, kidneys and liver) of rats.** (a) 6-month chronic treatment; (b) 9-month chronic treatment. Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

(a)




(b)

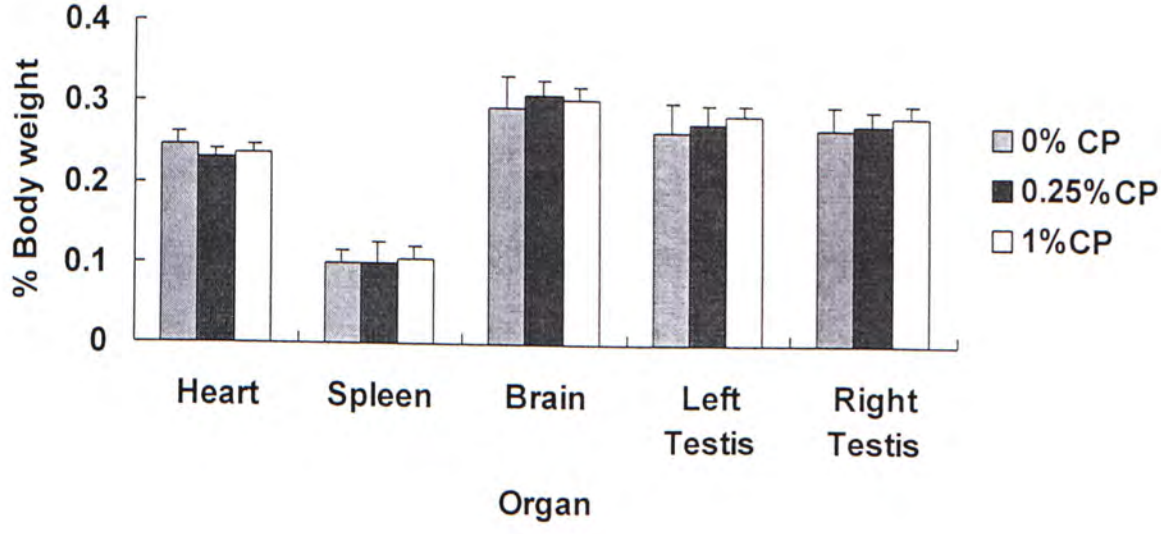
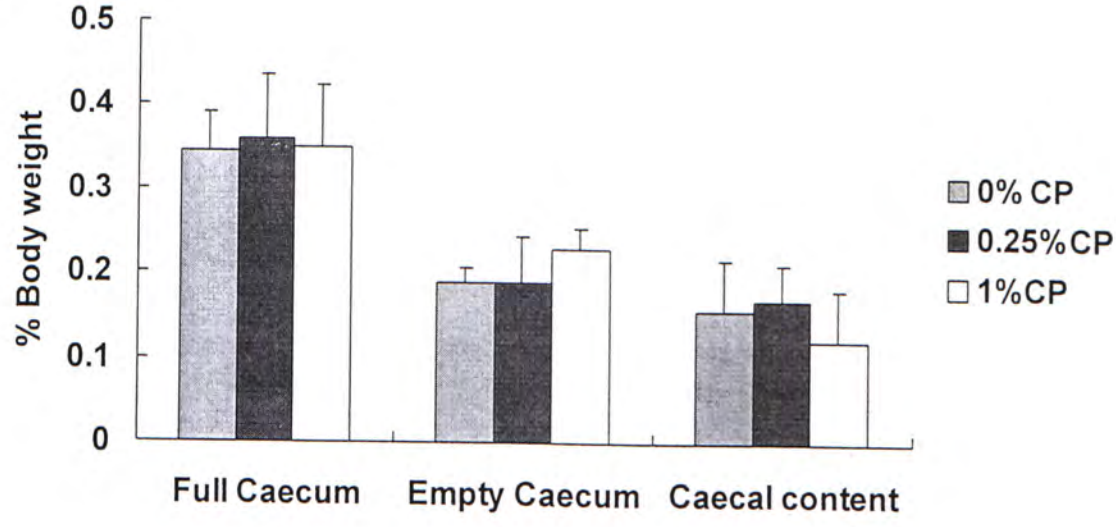



**Figure 3.29 Effect of chronic treatments of CP on the relative organ weights (heart, spleen, brain and testes) of rats.** (a) 6-month chronic treatment; (b) 9-month chronic treatment. Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

(a)




(b)

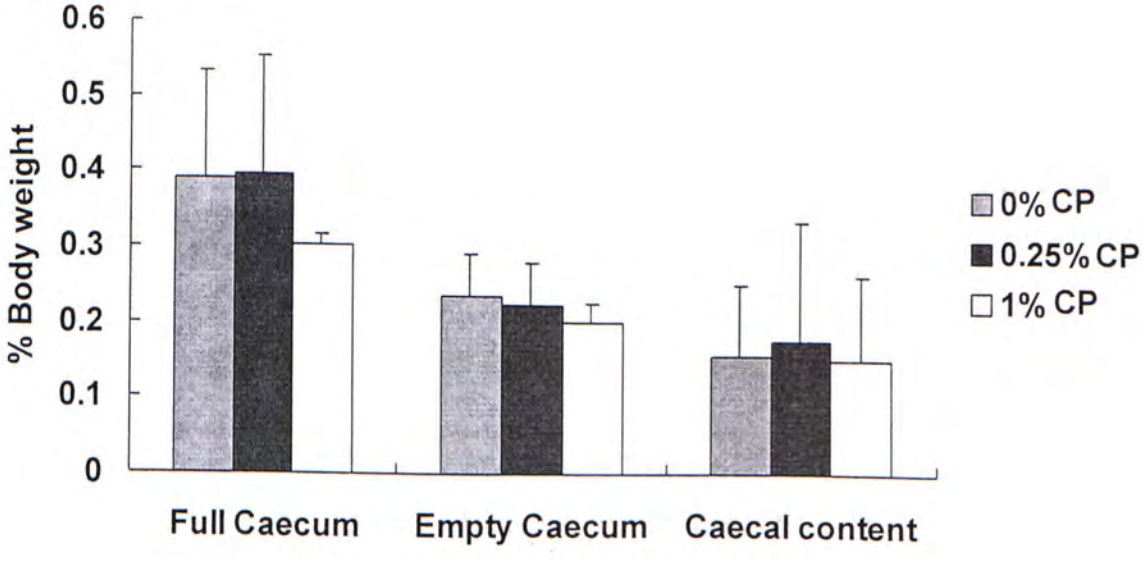



**Figure 3.30 Effect of chronic treatments of CP on the relative weights of caecal contents of rats.** (a) 6-month chronic treatment; (b) 9-month chronic treatment. Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

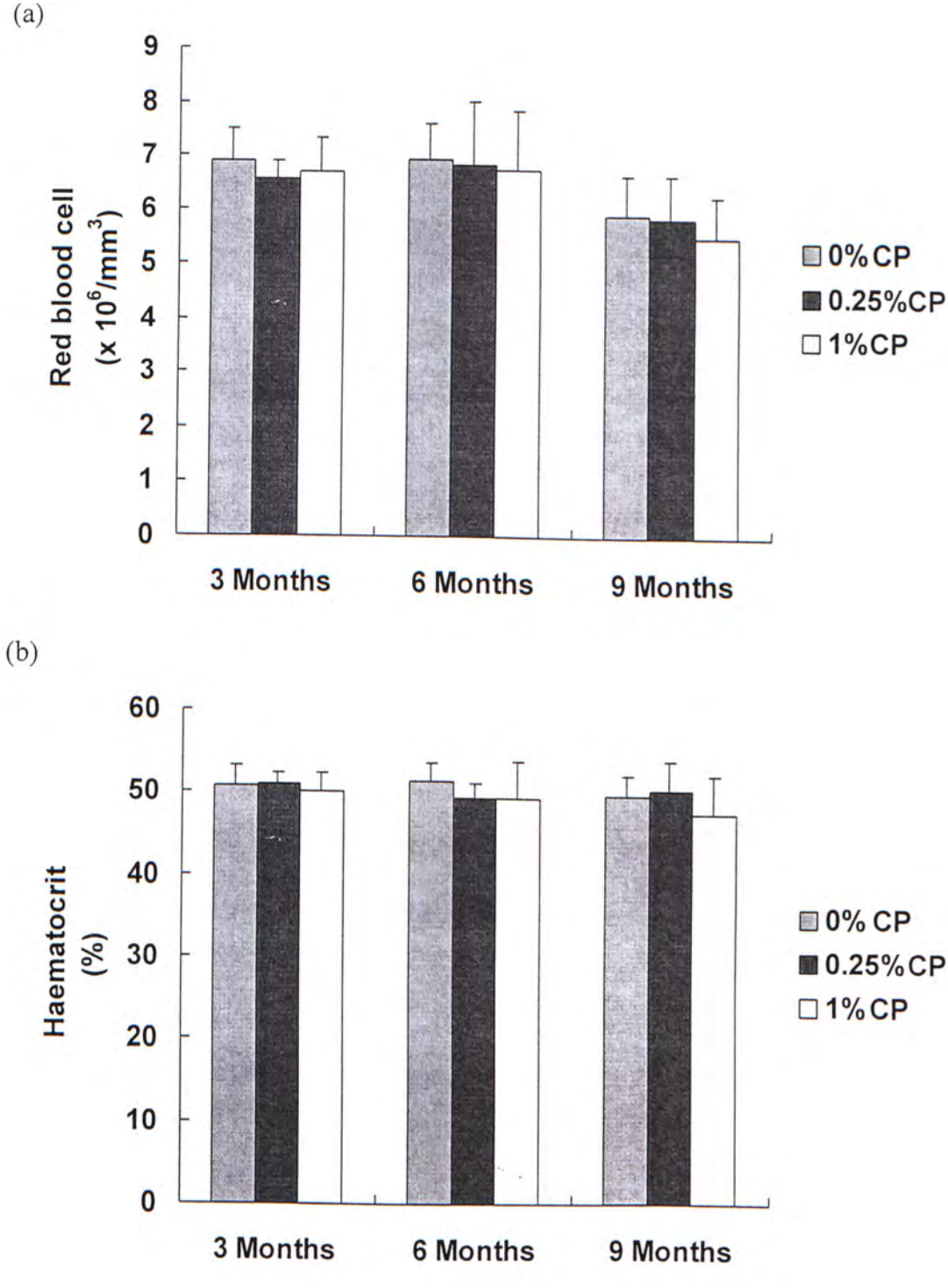



**Figure 3.31 Effect of chronic treatments of CP on blood chemistry (1) of rats.** (a) red blood cell counts; (b) haematocrit. Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

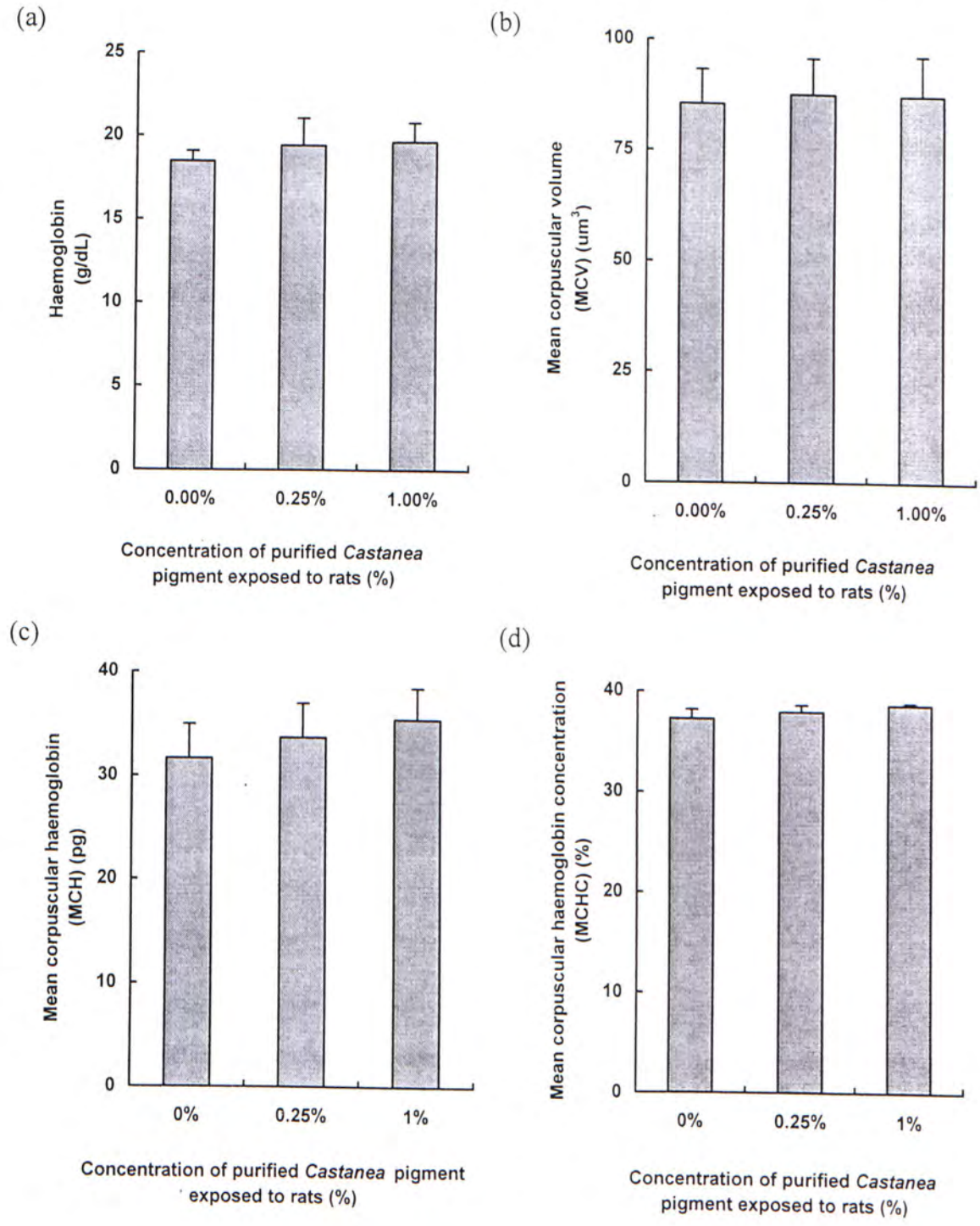



**Figure 3.32 Effect of 9-month chronic treatment of CP on blood chemistry (2) of rats.** (a) haemoglobin; (b) MCV; (c) MCH; (d) MCHC. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

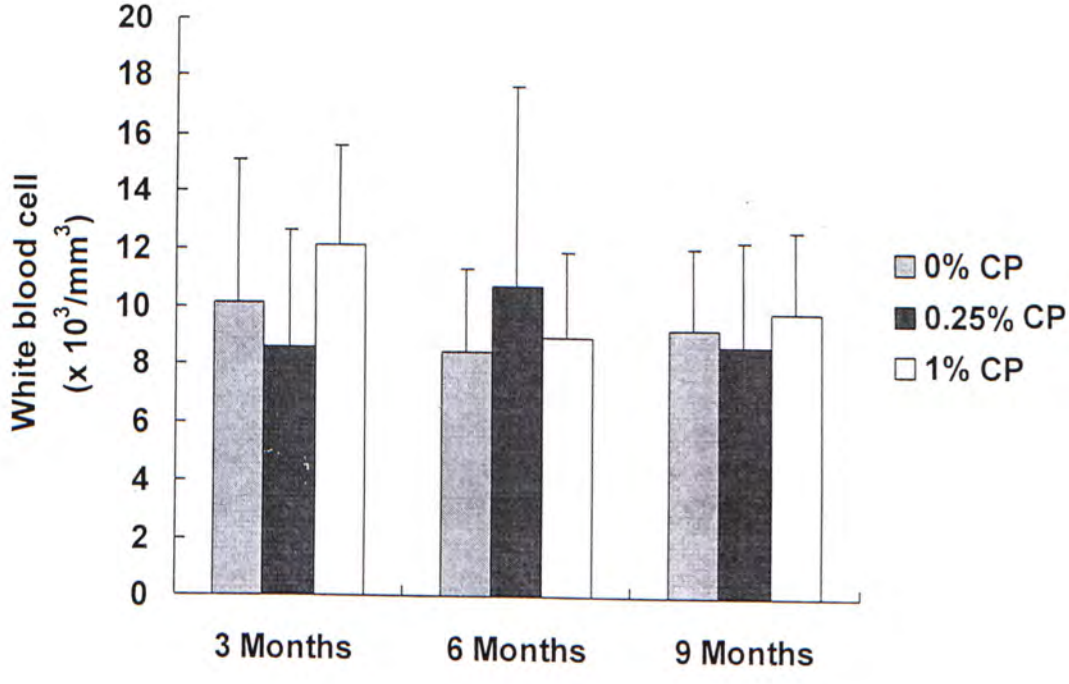
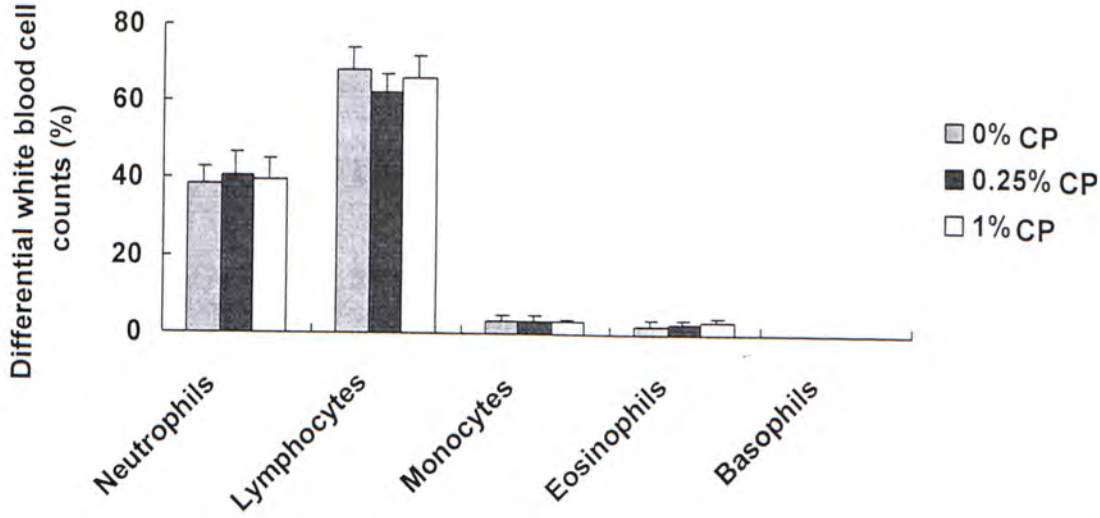



**Figure 3.33 Effect of chronic treatments of CP on the total white blood cell counts of rats.** Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

(a)




(b)

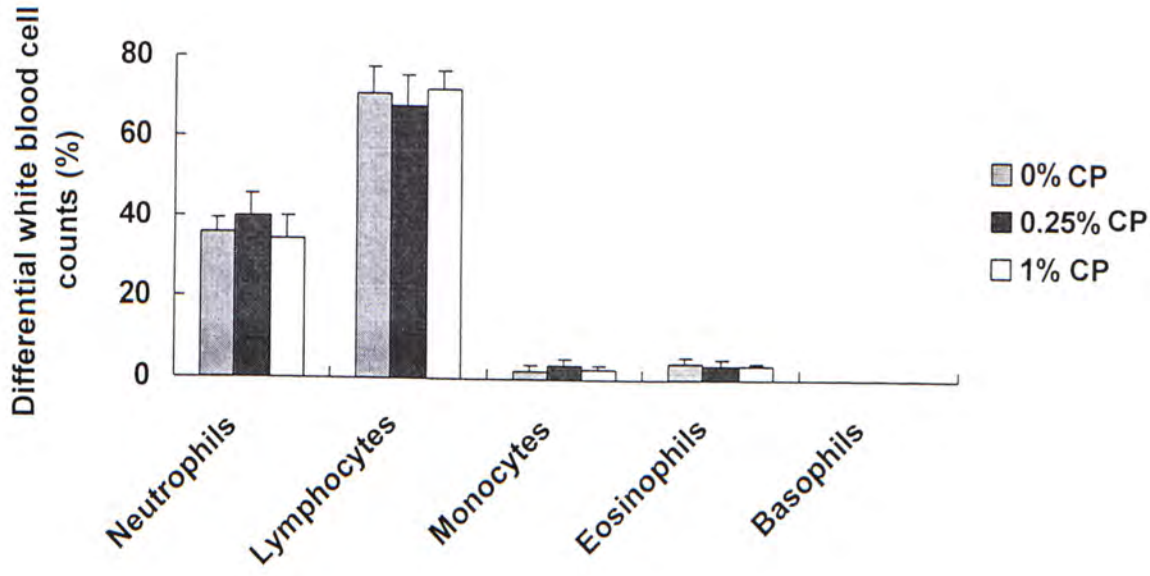



**Figure 3.34 Effect of chronic treatments of CP on the differential white blood cell counts of rats.** (a) 6-month chronic treatment; (b) 9-month chronic treatment. Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

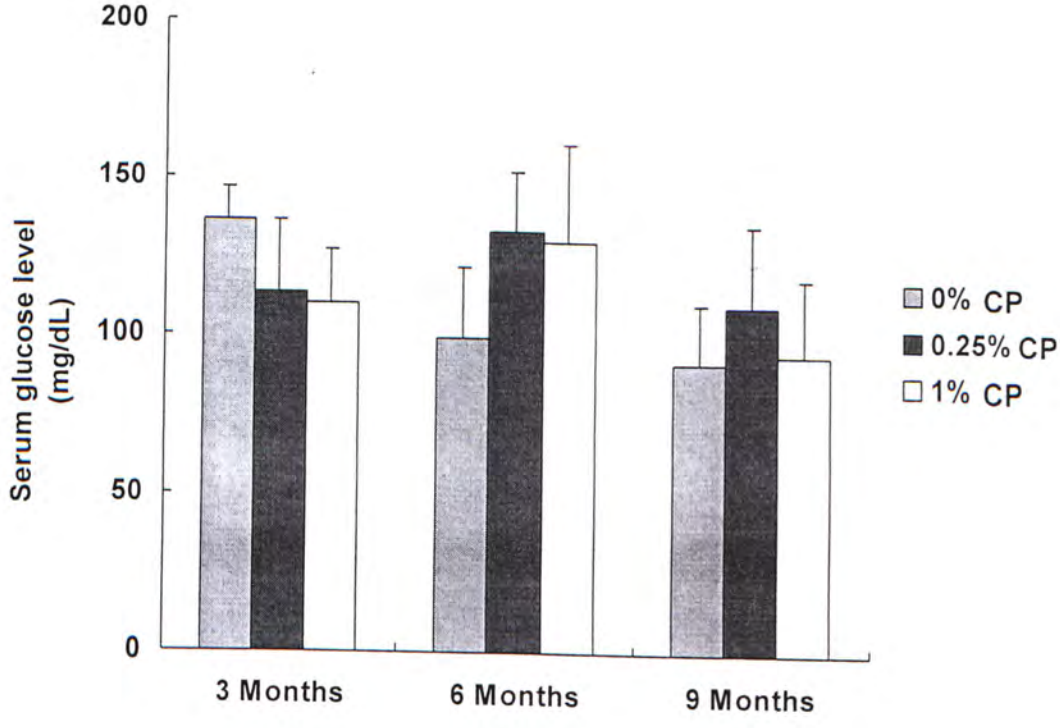
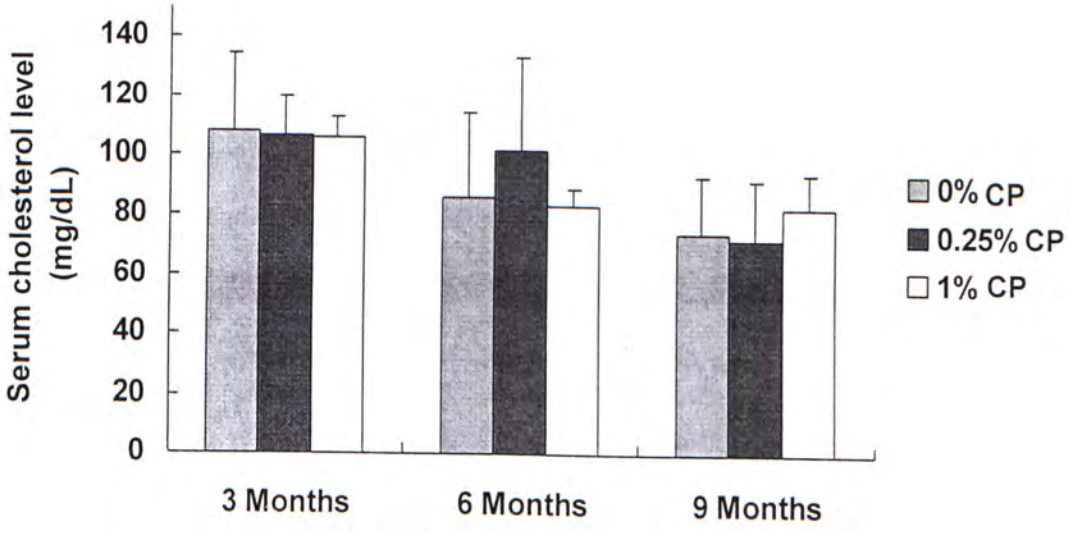



**Figure 3.35 Effect of chronic treatments of CP on serum glucose levels of rats.** Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

(a)




(b)

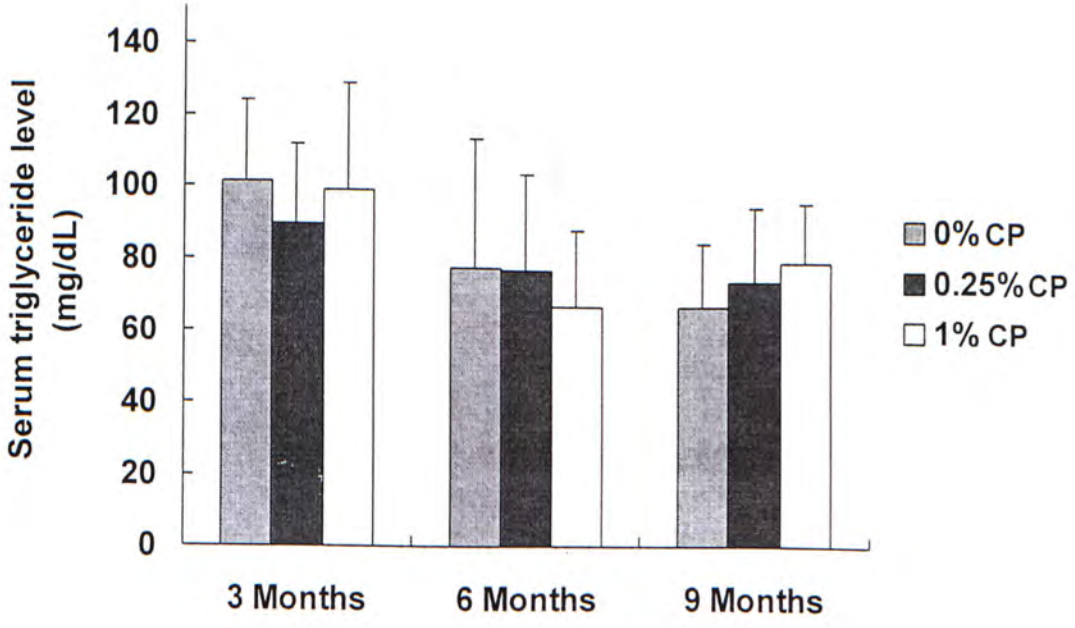
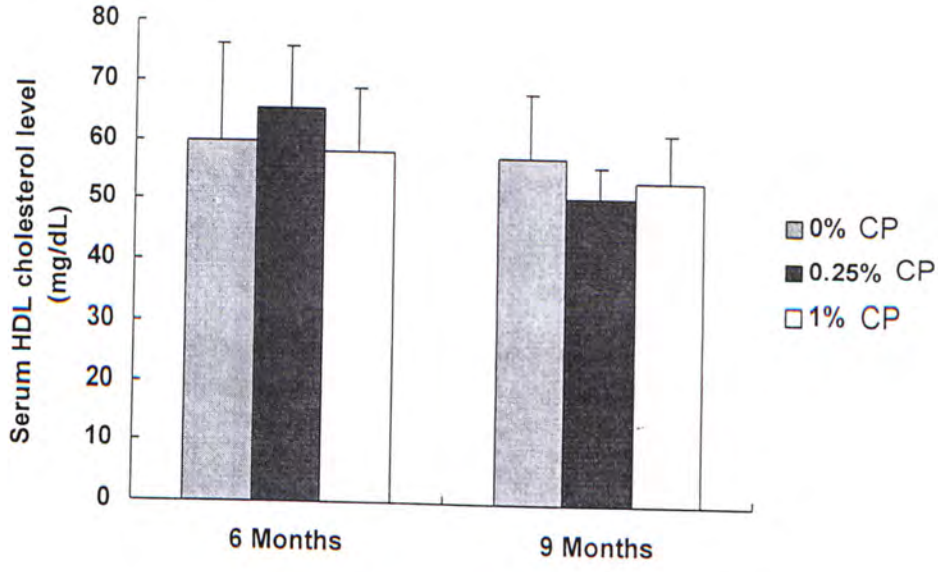



**Figure 3.36 Effect of chronic treatments of CP on serum lipids of rats.** (a) total cholesterol; (b) triglyceride. Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

(a)




(b)

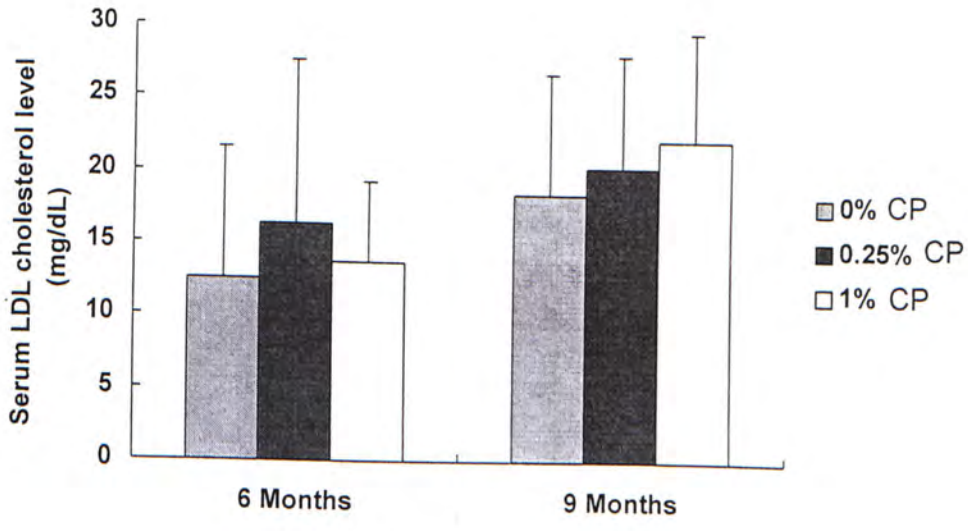



**Figure 3.37 Effect of 6-month and 9-month chronic treatments of CP on serum lipids.** (a) HDL cholesterol; (b) LDL cholesterol. Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

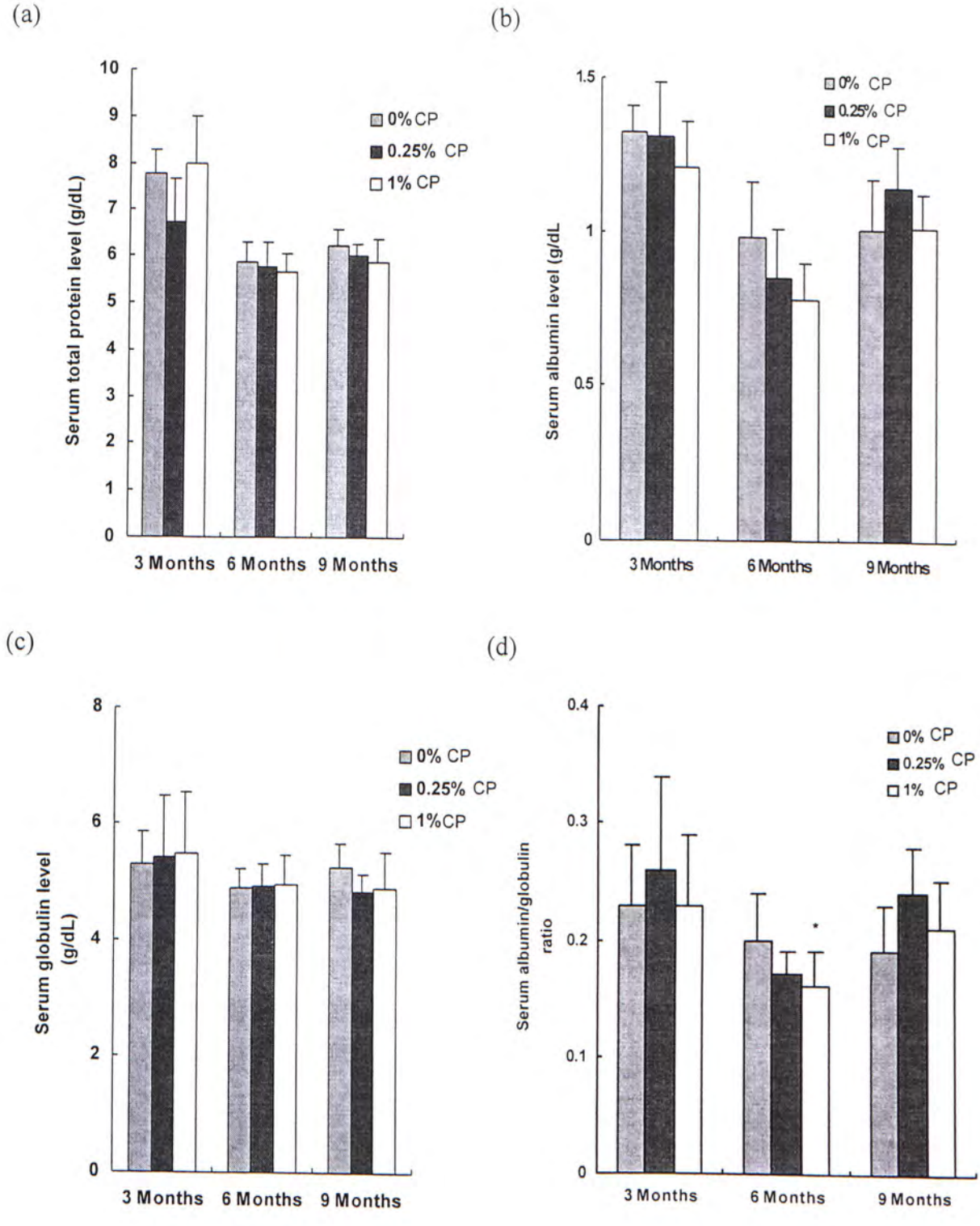
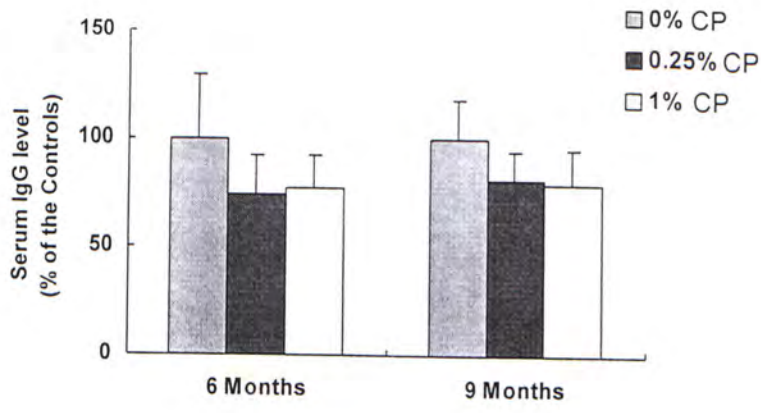
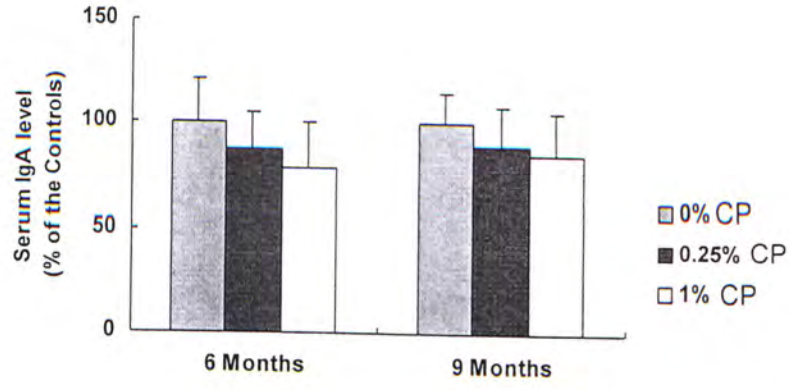



**Figure 3.38 Effect of chronic treatments of CP on serum proteins of rats.** (a) total protein; (b) albumin; (c) globulin; (d) albumin/globulin ratio. Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. * Results having no significant difference at $p<0.01$. All the other results had no significant difference ($p<0.05$).

(a)




(b)




(c)

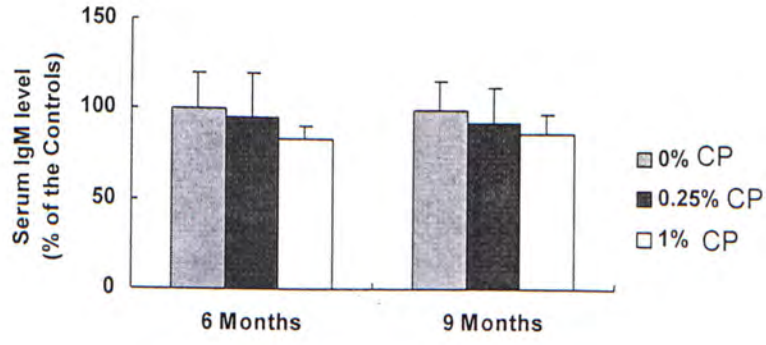
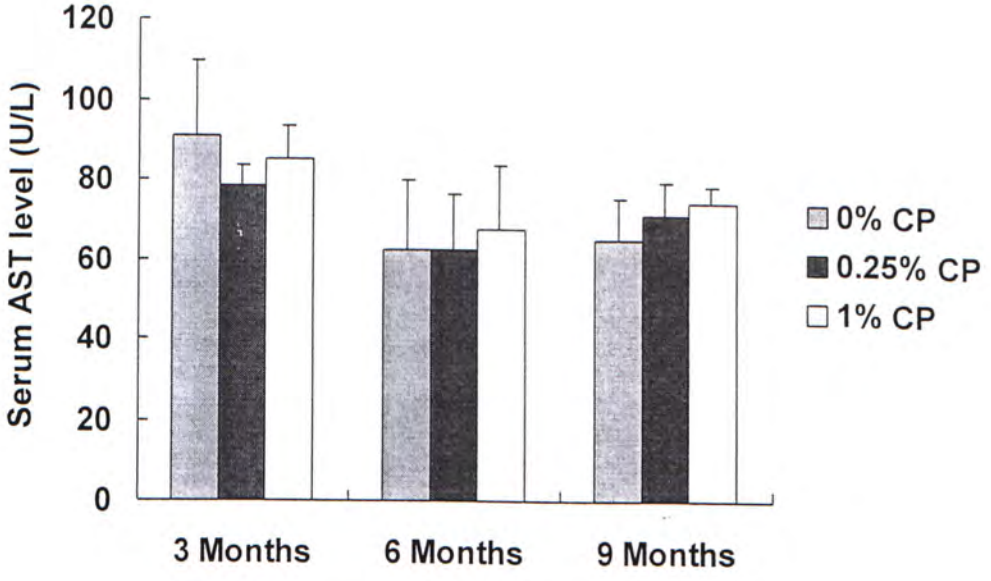



**Figure 3.39 Effect of chronic treatments of CP on serum immunoglobulins of rats.** (a) IgG; (b) IgA; (c) IgM. Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8 and were expressed as a percentage of the controls. All results had no significant difference ($p<0.05$).

(a)




(b)

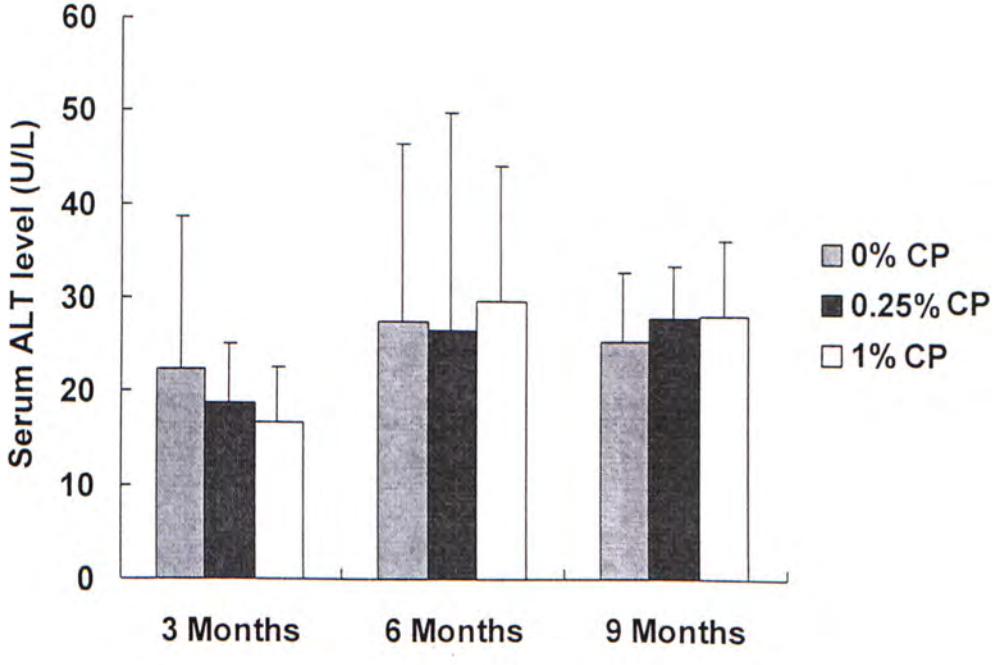
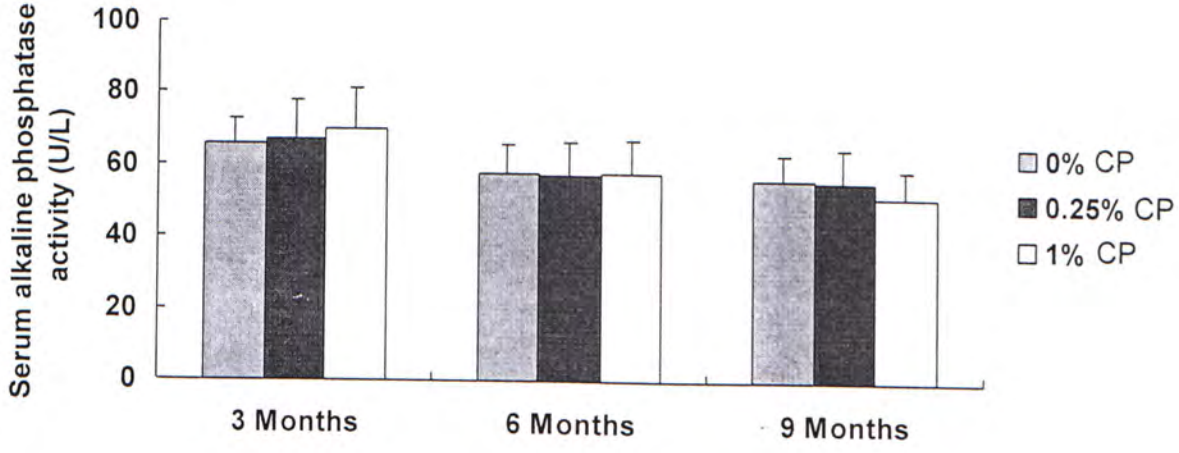



**Figure 3.40 Effect of chronic treatments of CP on serum aminotransferase of rats.** (a) AST; (b) ALT. Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

(a)




(b)

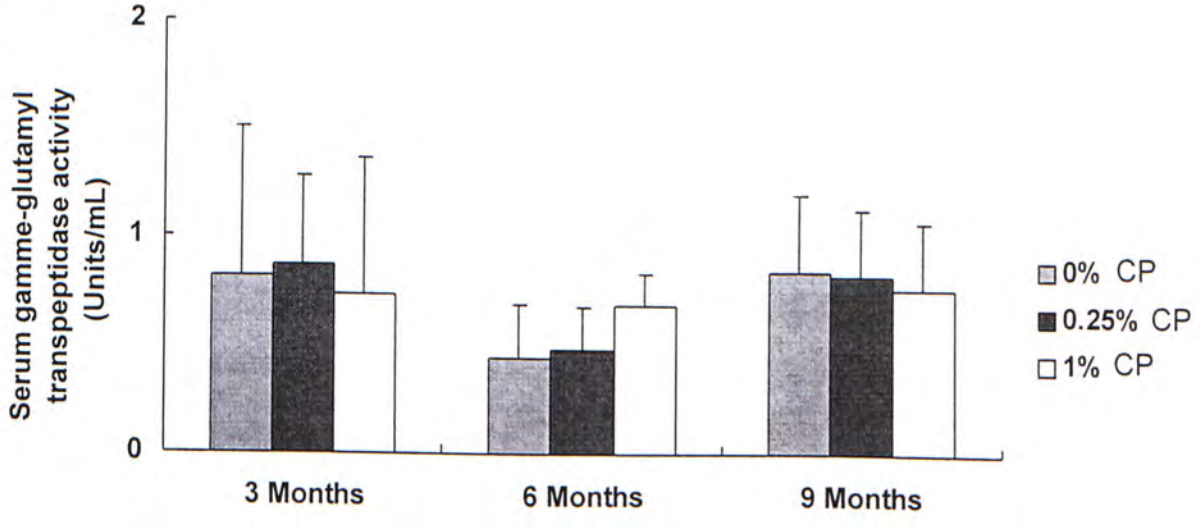



**Figure 3.41 Effect of chronic treatments of CP on serum liver enzymes of rats.** (a) alkaline phosphatase; (b) γ-glutamyl transpeptidase. Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

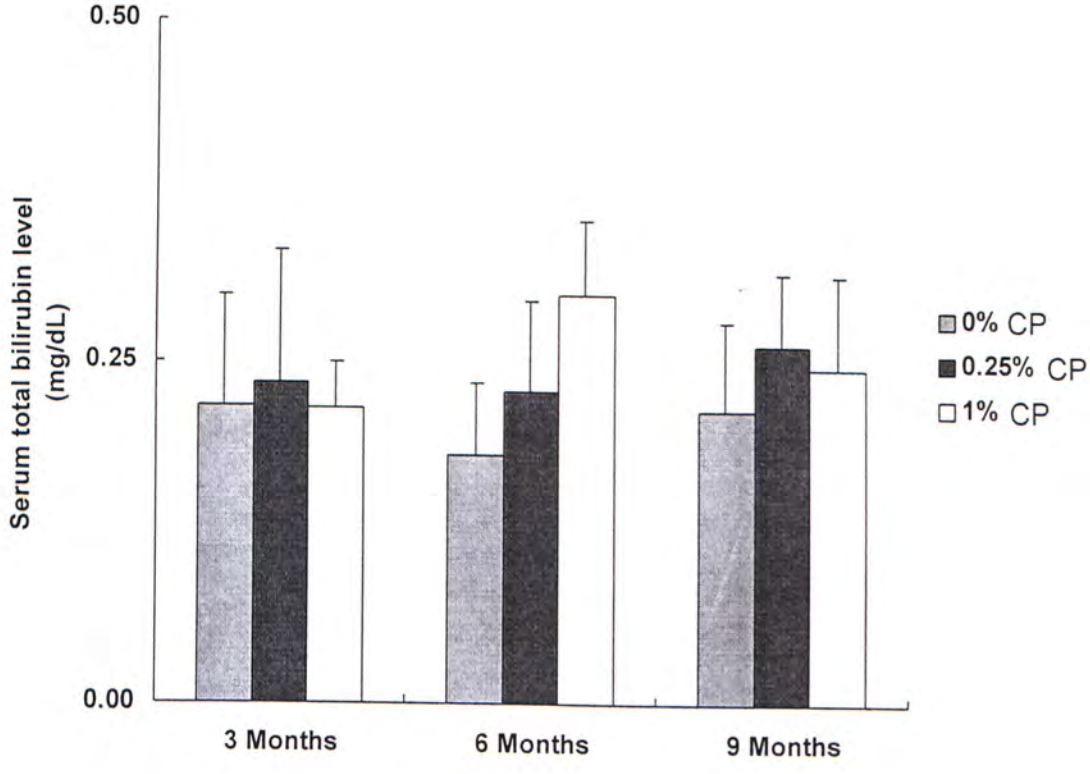



**Figure 3.42 Effect of chronic treatments of CP on serum total bilirubin levels of rats.** Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

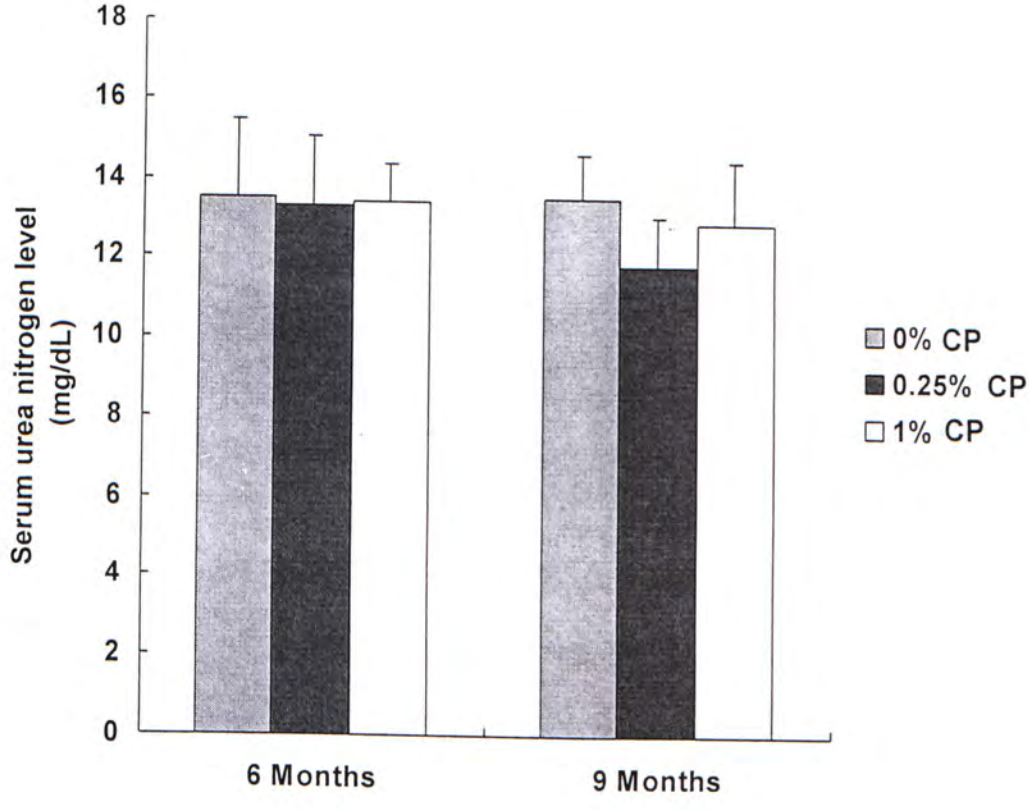
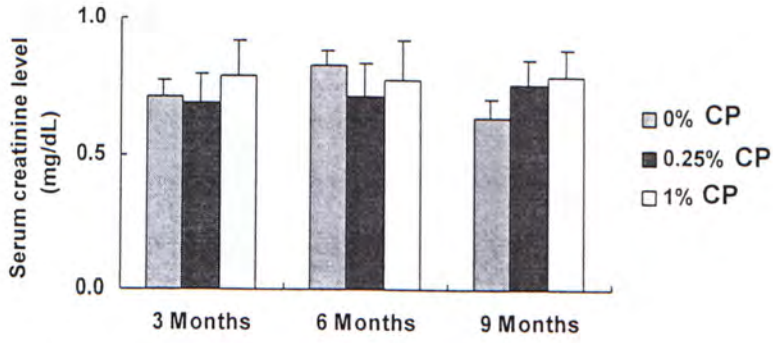
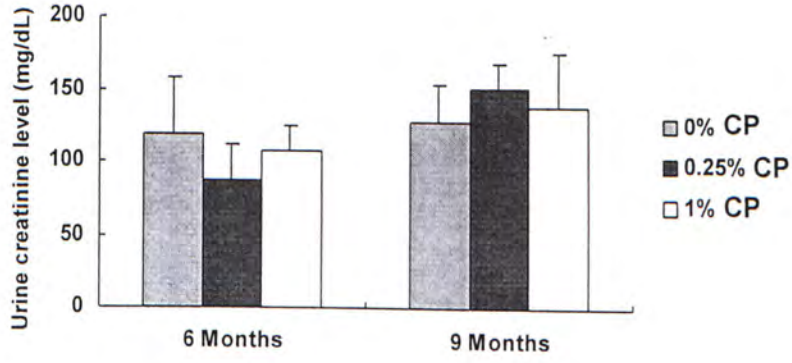



**Figure 3.43 Effect of 6-month and 9-month treatments of CP on serum urea nitrogen levels of rats.** Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

(a)




(b)




(c)

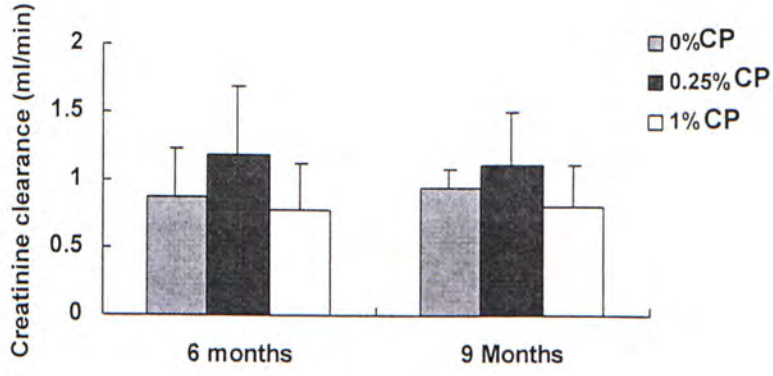
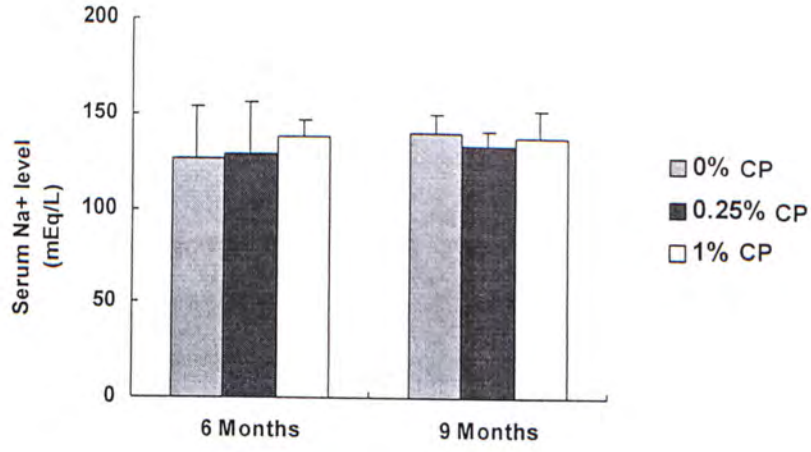
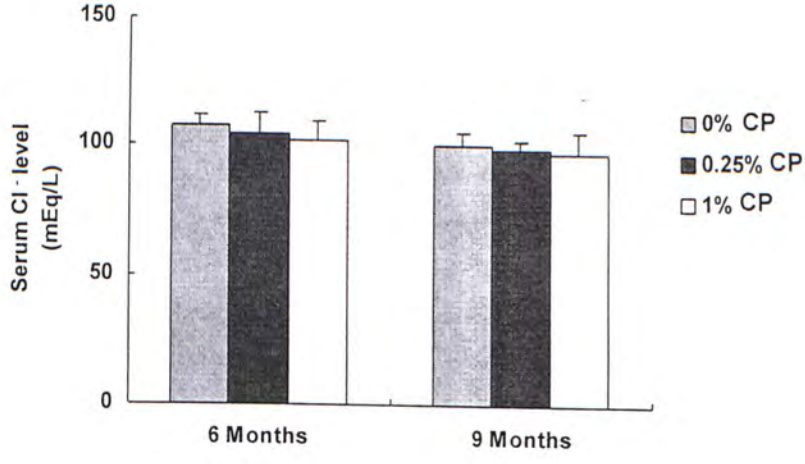



**Figure 3.44 Effect of chronic treatments of CP on creatinine of rats.** (a) serum creatinine; (b) urine creatinine; (c) creatinine clearance. Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

(a)




(b)




(c)

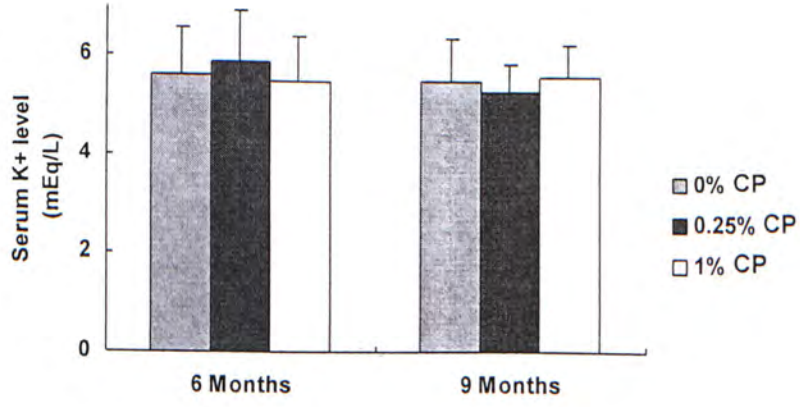
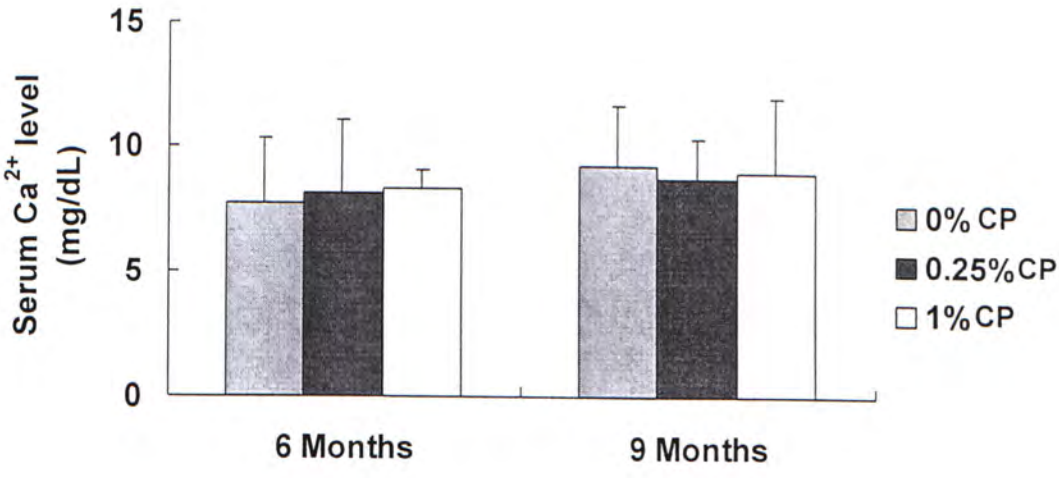



**Figure 3.45 Effect of chronic treatments of CP on monovalent ions of rats.** (a) sodium ion; (b) chloride ion; (c) potassium ion. Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

(a)




(b)

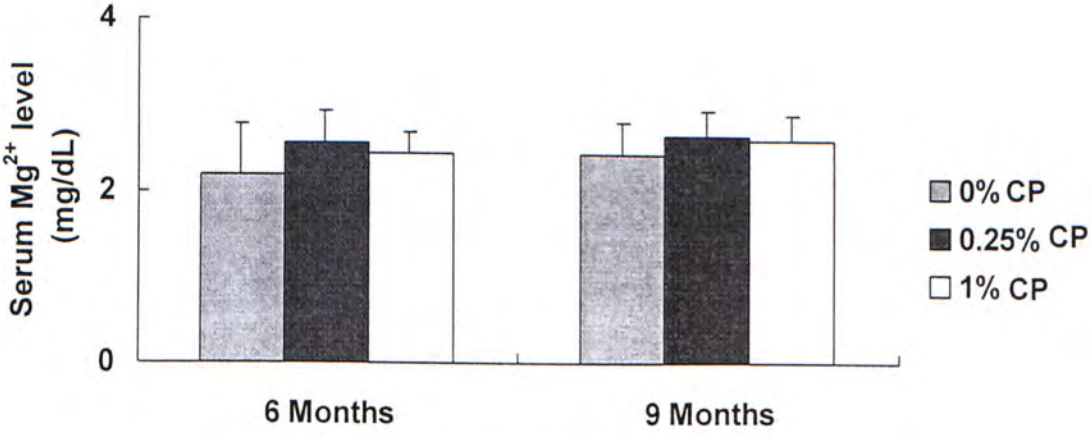



**Figure 3.46 Effect of chronic treatments of CP on divalent ions of rats.** (a) calcium ion; (b) magnesium ion. Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. All results had no significant difference ($p<0.05$).

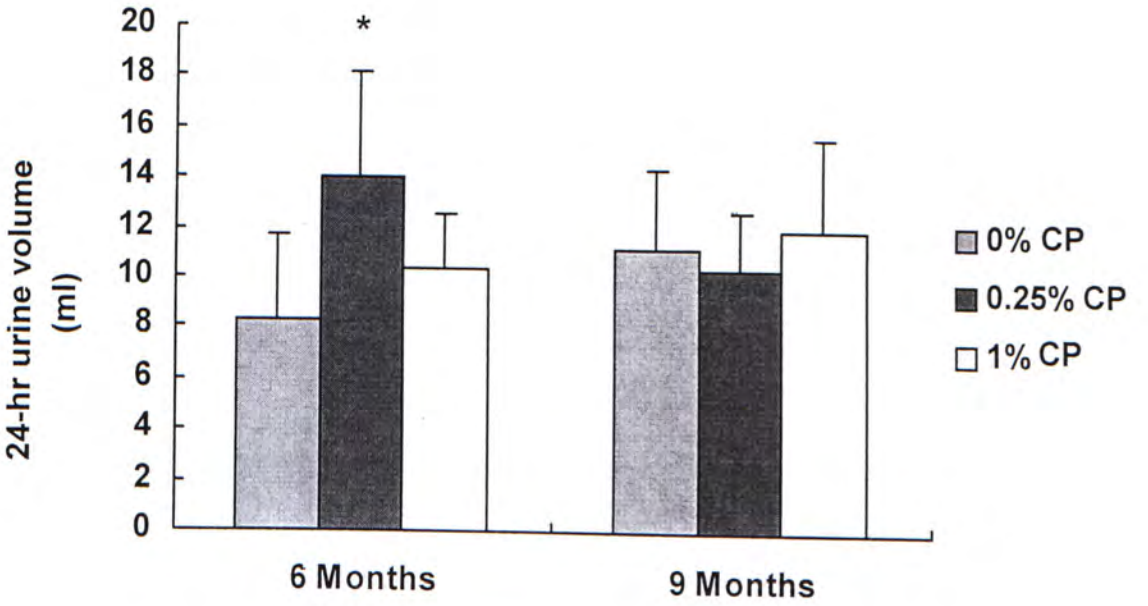



**Figure 3.47 Effect of chronic treatments of CP on 24-hr urine volumes of rats.** Concentration of purified CP exposed to rats were 0%, 0.25% and 1%. Results were means ± SD, no. of rats per treatment = 8. Significantly difference comparing with the control at $p<0.05$.

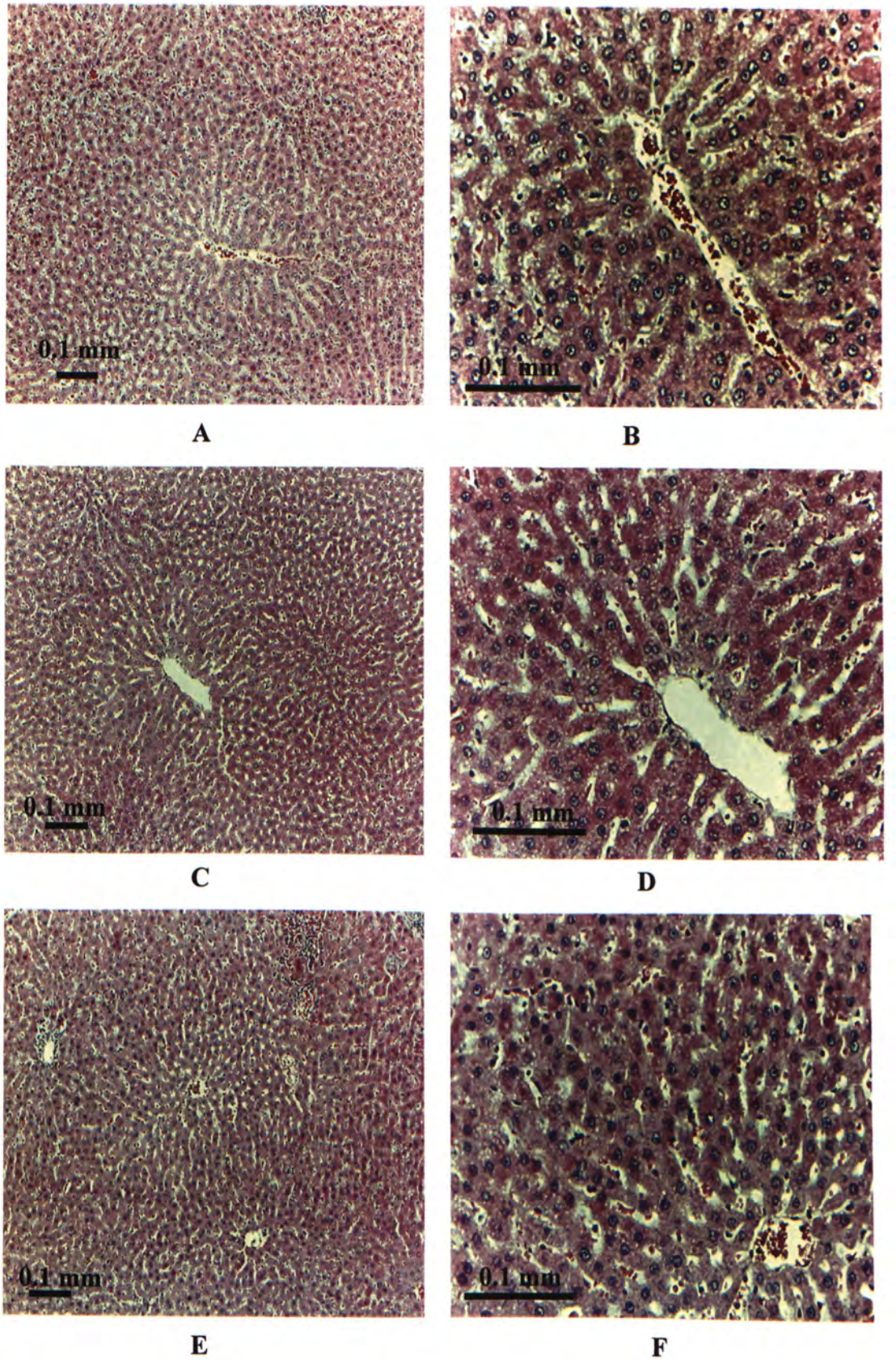
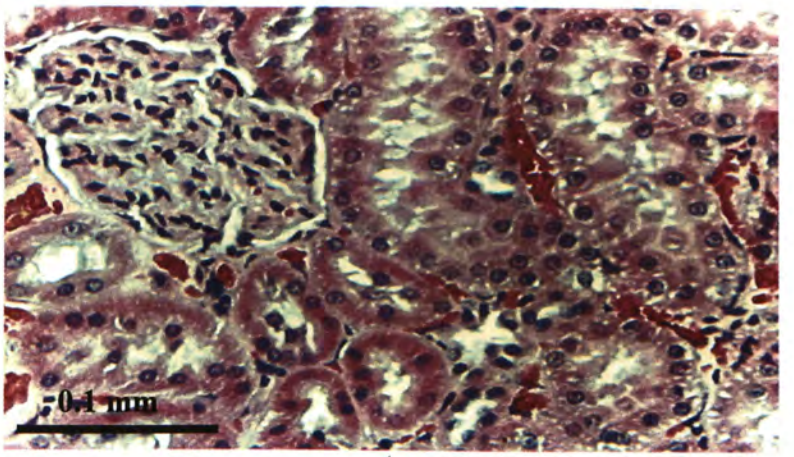
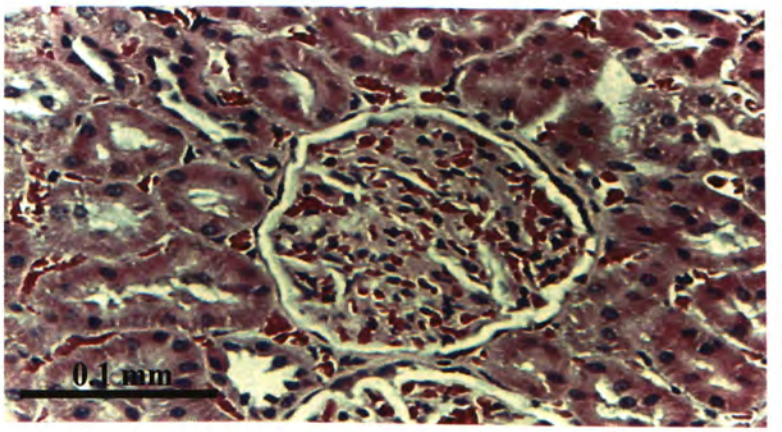


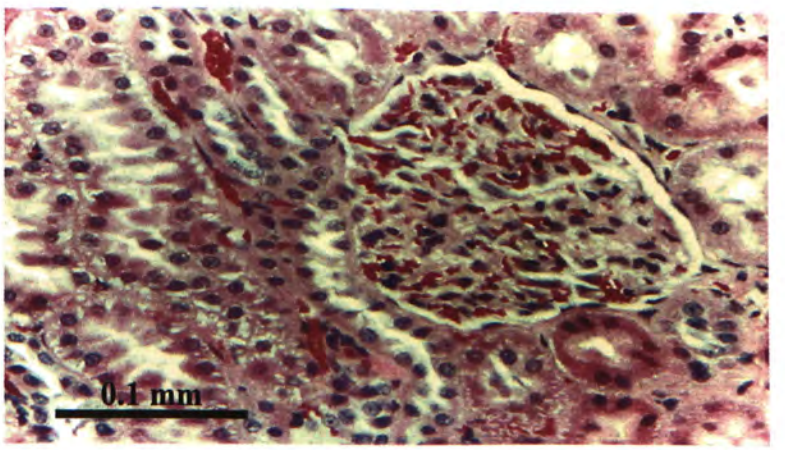

**Figure 3.48 Photomicrographs of liver sections from rats of the 9-month chronic toxicity test (H&E).** A. & B. Control;. C. & D. 0.25% CP; E. & F. 1% CP.




A




B




C

**Figure 3.49 Photomicrographs of kidney sections from rats of the 9-month chronic toxicity test (H&E).** A. Control; B. 0.25% CP; C. 1% CP.

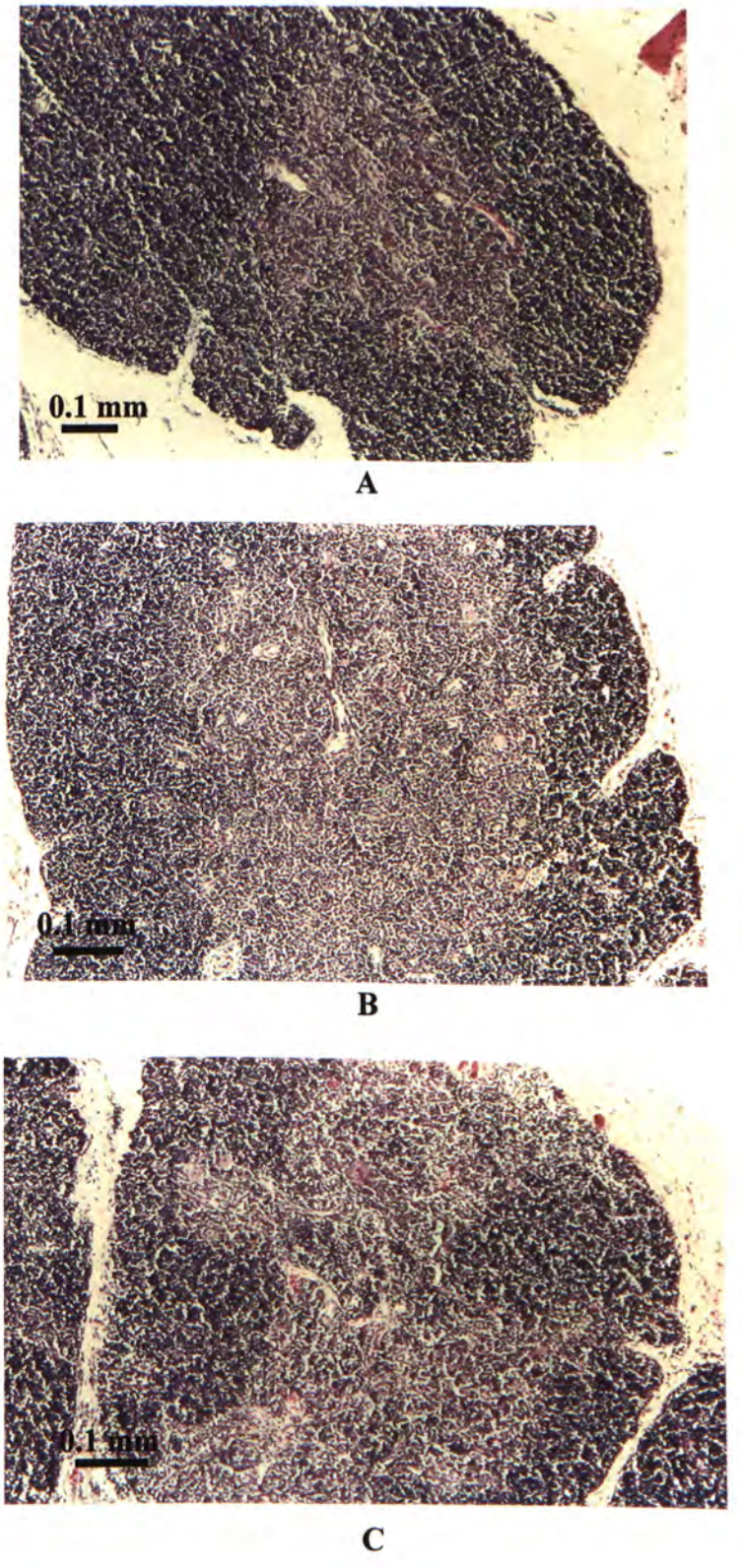



**Figure 3.50 Photomicrographs of thymic sections from rats of the 9-month chronic toxicity test (H&E).** A. Control; B. 0.25% CP; C. 1% CP.

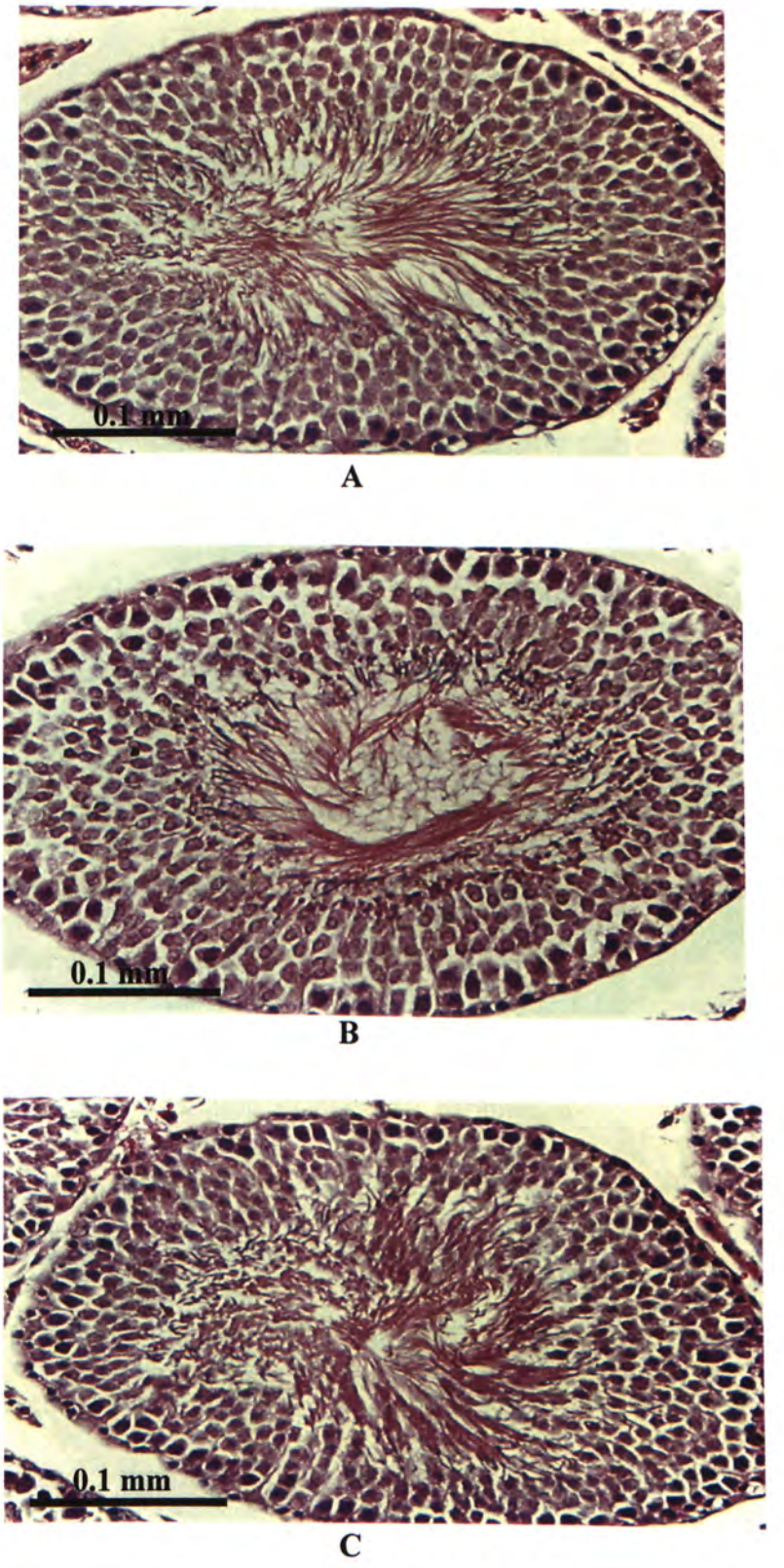



**Figure 3.51 Photomicrographs of seminiferous tubule of testis sections from rats of the 9-month chronic toxicity test (H&E).** A. Control; B. 0.25% CP; C. 1% CP.

# Chapter 4 Discussion

## 4.1 Pigment characterization

Stability is a very important factor for a pigment to be a potential natural colorant. The natural pigments associated with most fresh food, especially fruits and vegetables, are vivid and brilliant. However, these pigments are subject to adverse physical and chemical conditions during processing that cause their partial degradation. Processing therefore often results in undesirable changes in color and thus diminishes the visual perception of foods. Food manufacturers and processors thus require to replace the lost color if the acceptable attractive appearance is to be restored (Ghorpade *et al.*, 1994).

Synthetic color additives such as Allura Red and Sunset Yellow are widely used due to their coloring properties, uniformity, stability and low cost, although health problems occur after prolonged use (Goyle and Gupta, 1998).

A variety of beers, biscuits, gravy powders, confectionery products and baked foods were made with the addition of class III caramel, evidence showed that a marker component of the caramel was only partially stable during the manufacture, packaging and storage of the foods (Coffey *et al.*, 1997).

In this study, CP was found to be very stable. High temperature at 121 °C (autoclaving temperature) did not alter the spectrum of CP solution (Figure 3.1a). Moreover, there was no significant effect ($p<0.05$) on the absorbance of CP under various conditions for a period of 12 months (Figure 3.2). Furthermore, pH profile of CP was found to be stable (Wong, unpublished data).

As the consumers' concern on the safety of synthetic colorants remains and the demand for natural pigments as food colorants has increased, a natural and stable CP is therefore suitable to be used in the food industry to enhance the aesthetic appeal of

processed food. For instance, as CP was found to be heat and light stable, these two properties allow CP to be a food colorant which can be applied on canned food, baking products and gravy powders.

In the purification of CP, the solvents dichloromethane and ethyl acetate were used to remove the impurities in the crude pigment. HPLC has been commonly used to isolate and identify pigments such as carotenoids (Deli *et al.*, 1996), anthocyanins (Yoshida *et al.*, 1996; Rodriguez *et al.*, 1998) and synthetic colorants (Virtanen *et al.*, 1999). Further purification of CP by reversed-phase HPLC was carried out. From the HPLC profile shown in Figure 3.3, a major peak at retention time of 19 minute was detected. The collected fraction was light brown in color. This indicated that one major pigment attributes to the pigmentation of chestnut shells. The spectrum of HPLC-19 minute fraction of CP (Figure 3.1b) was different from that of purified CP (Figure 3.1a). The former showed much less absorption in the UV light region (200-400 nm) than the latter. This further suggested that HPLC-19 min. fraction of CP contained less impurities and therefore was much more pure than the purified CP which was solely purified by the process of solvent extraction using dichloromethane and ethyl acetate.

BHA and BHT are free radical scavengers and have been shown to possess strong antioxidative activities in many studies (Camire and Dougherty, 1998; Iverson, 1999). However, their potent tumor-promoting effects (Lindenschmidt *et al.*, 1986) have aroused public rejection. On the contrary, vitamin C (ascorbic acid), vitamin E (α-tocophero) (Tsao, 1997) and phenolic compounds such as catechin, quercetin and caffeic acid (Chen and Ho, 1997) have been intensively used for food additives nowadays due to their high antioxidative activities and their natural in origin.

Antioxidants can be classified into three categories based on their reaction mechanisms: (1) preventing radical formation either by chelation of transition metal

ions or by inhibition of enzymes that generate free radicals (Kono *et al.*, 1998); (2) scavenging free radicals once they formed (Salah *et al.*, 1995); and (3) acting as synergists with other antioxidants (Jia *et al.*, 1998). The results obtained in the $DPPH^{\bullet}$ scavenging assay indicated that CP exhibited strong antioxidative activity as they can scavenge the free radical (Figure 3.4). The antioxidative activity of a 0.1% CP solution was equivalent to that of 25 mM BHA. The CP purified by HPLC had a much lower antioxidative activity. Only about 25% of the antioxidative activity remained in the HPLC-purifed pigment. This suggests that both CP and other impurity compounds have such activity.

For the applications of CP in the food industry, CP is not only a natural colorant but also can be used as a food preservative likes BHA and BHT. CP can be added to food products to extend the shelf life by preventing lipid peroxidation.

## 4.2 Toxicological studies of CP

More and more synthetic food coloring additives, after prolonged use, have been found to have health problems such as indigestion, anaemia, pathological lesions in brain, kidney, spleen and liver, tumours and cancer, paralysis, mental retardation, abnormalities in offspring, growth retardation, and eye defects resulting in blindness. It is recommended that these hazardous additives should be avoided to use in food (Aboel *et al.*, 1997; Goyle and Gupta, 1998). Natural colorants would be good candidates to replace the synthetic ones.

In this project, toxicological studies of CP were conducted both on bacteria (Microtox® test and Mutatox® test) and rats (acute toxicity test and chronic toxicity test).

Microtox® test has been used to assess the toxicity of over 1,300 chemicals (Kaiser and Palabrica, 1991). It is mainly used for testing environmental toxicity. In this study, Microtox® test was used as a preliminary test on the toxicity of CP. The procedures are simple, inexpensive and data can be obtained within a short period of time. In general, EC50 of a sample at 2.0 ppm (0.002 mg/ml) was considered to be highly toxic (Hong and Pehkonen, 1998). The EC50 values of purified CP and crude CP were found to be 650 ppm (0.65 mg/ml) and 360 ppm (0.36 mg/ml) respectively. These high EC values suggest that CP is relatively non-toxic and safe to be included in food items. In addition, purification using dichloromethane and ethyl acetate was able to remove toxic impurities that present in the crude pigment because the purified CP had a higher EC50 value than that of the crude one (Figure 3.6).

Like Microtox® test, Mutatox® test was used as a screening or preliminary test on the mutagenicity of CP. The principle of Mutatox® test is based on the Ames test. Although it does not necessarily follow that because a compound is mutagenic

it is also carcinogenic, the correlation is quite high. Thus, the knowledge that a compound is mutagenic in a bacterial system serves as a warning of possible danger (Madigan *et al.*, 1997). Mutatox® test has been used in the analyses of fatty acid derivatives (Sun *et al.*, 1998) and environmental samples of landfill leachates (Beg and Al, 1998). In this study, Mutatox results suggested that CP was not a mutagenic agent (Table 3.1).

In the 14-day acute toxicity study, a high dosage of 8% CP in the drinking water was used to feed the rats. The daily uptake of CP of a treated rat was 7143 mg/kg body weight which was more than 35 times to the daily maximum uptake of caramel, 200 mg/kg body weight (Chappel and Howell, 1992).

No death or adverse effect was observed during this study. Physical parameters including body weights, food and fluid consumption were normal for control and treated rats. Comparably, there were also no treatment-related ante-mortem observations of the above parameters in the rats having a high consumption of class III caramel or ammonia caramel (AC) in a 4-week toxicity study (MacKenzie *et al.*, 1992).

For the relative organ-weight data, there was no significant difference ($p<0.05$) in all the organs except the testes. This difference was, however, insignificant at $p<0.01$ (Figure 3.8b), which might be only due to the generally large variations among the individuals. For the relative caecal contents of the two groups (Figure 3.9), addition of 8% CP to the drinking water of the treatment group significantly increased ($p<0.05$) the relative caecal content. Located at the junction of the colon and the ileum, the caecum of the rat was the blind end of which forms the appendix. It contains symbiotic bacteria and protozoa which secrete cellulase to digest cellulose which is a tough fibrous carbohydrate found in plant cell walls (Rowett, 1974). The rats of the treatment group consumed enormous amounts of plant components,

containing in the 8% CP solution. These rats were trying to adapt during the 14-day period. Hence, it was possible that the increase in caecal content might be due to the large quantities of cellulose, the plant components and the symbiotic bacteria being induced by the CP.

In the 6-month and 9-month chronic treatments, mean body weight gains, fluid consumption and relative organ-weight of rats were not affected by feeding CP under the conditions of this study. Unlike the 14-day acute test, there was no significant difference in the caecal contents among the groups (Figure 3.30). Therefore, the increase in caecal contents did not occur in the rats which have adapted to the ingestion of CP during the long period of treatment although they received a relatively low concentration of CP, i.e. 0.25 % and 1 %, in the chronic treatments.

In the case of AC- and THI-exposed rats, it was found that there was a slight decrease in the absolute liver weight (to 89%; $p<0.05$) and absolute thymus weight (to 89%; $p<0.01$). Moreover, the relative kidney weights were increased by 8% ($p<0.05$) and the relative weights of spleen were significantly ($p<0.05$) decreased to 71% (Houben *et al.*, 1992).

The haematology results of the acute and chronic toxicity studies in this project demonstrated that the effect of CP in the rats was negligible. The treatment rats had normal red-cell count, haematocrit, total white-cell count and differential white-cell count. Therefore, by the study of the composition of the blood and their production, the blood in rats of the treatment group was found functioning normally.

In the case of caramel, there were a number of significant differences ($p<0.05$) in the treatment group with a high consumption of class III caramel. Total leukocyte numbers of AC- and THI-exposed rats were decreased to about 34% of the control counts, due to a significant reduction in the number of blood lymphocytes to about 26% (Houben *et al.*, 1992). Total numbers of spleen cells in AC- and

THI-exposed rats were also decreased to 47% and 43% of the control counts respectively (Houben *et al.*, 1992). Cell numbers isolated from popliteal lymph nodes were decreased to 38% and 39% in AC- and THI-exposed rats respectively (Houben *et al.*, 1992).

For the rats having a high dosage of CP, like caramel (MacKenzie *et al.*, 1992), there was no significant difference in serum glucose, total cholesterol, triglycerides, HDL cholesterol and LDL cholesterol levels when compared to the control group. One point to note, the fasting blood glucose amount in the treatment group of the acute study was slightly lower than the given reference range (Figure 3.12). This leads to a further question that whether CP can reduce the glucose level in rats. Nevertheless, there was no elevation of glucose and lipids, hence indicating that a high dosage of CP did not have any harmful effects to the cardic system of the rats.

The assessment of proteins can provide an overall picture of protein homeostasis, in particular, the immunoglobulins, which are important in the immune system. In the acute test, there was no significant difference ($p<0.05$) found in measurements of serum total protein, globulin, A/G ratio and immunoglobulins of the two groups. In the 6-month chronic treatment, 1% CP-exposed rats had a significantly lower ($p<0.05$) A/G ratio when compared to the control group, but insignificant at $p<0.01$ (Figure 3.38d). The A/G ratio indicates the balance between the total albumin and total globulin and is usually evaluated in relation to the total protein level (Cella and Watson, 1989). In this study, there was no significant difference ($p<0.05$) in the total protein levels of the experimental rats (Figure 3.38a). The intake of caramel, like CP, can induce no significant difference ($p<0.05$) for most of the factors tested above (MacKenzie *et al.*, 1992). However, it was found that the total IgA levels in the ammonia caramel (AC)-exposed rats were decreased to 49% of control rats (Houben *et al.*, 1992).

In the liver function test, like caramel (MacKenzie *et al.*, 1992), several enzymes including aspartate transferase (AST), alanine transferase (ALT), alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (GGTP), and total bilirubin in serum of the rats were examined. Changes in amount of these substances implies liver malfunction.

AST and ALT are the two enzymes commonly assayed for evaluating liver cell damage. Normally, the increase in AST level is associated with the liver cell damage, hepatitis and/or myocardial infarction. With mild hepatocellular damage, ALT levels are higher than AST while the elevation of the latter was found in more severe cellular necrosis due to the release of mitochondrial enzymes (Walmsley and White, 1994). In the acute study, there was however, only a significant reduction ($p<0.05$), not elevation of AST level in the treatment group (Figure 3.16). The decrease in AST level may indicate the decreased hepatocellular production or release. However, it was not a pathologically important phenomenon (Hall, 1992). On the contrary, CP intake may, on the other hand, have a protective function to the hepatic systems of the rats.

GGTP level in the 8% CP-exposed rats was significantly ($p<0.05$) lower than that of the control group (Figure 3.17b). Most GGTP in serum derives from hepatobiliary sources and elevated levels lead to hepatobiliary disease (Cella and Watson, 1989). Like AST, the reduced level of GGTP in the treated rats does not indicate any harmful effects.

Serum total bilirubin and the other enzymes such as ALT and ALP which were also tested for AC (MacKenzie *et al.*, 1992), had no significant treatment-related changes ($p<0.05$).

By measuring these factors in the liver function test, application of 8% CP in the treatment group did not cause any adverse effect to their hepatic systems.

In the renal function test, blood urea nitrogen, serum creatinine, urine creatinine and creatinine clearance levels were similar and normal in both of the control and treatment groups. Creatinine clearance is a sensitive indicator of glomerular function and renal disease is the major cause of reduced creatinine clearance (Cella and Watson, 1989). No significant difference ($p<0.05$) was detected for all the renal parameters. Thus, all rats had good functions of kidneys.

Electrolytes are substances that dissociate into electrically charged ions when dissolved. Both cations (sodium, potassium, calcium and magnesium) and anions (chloride) affect the electrical and osmotic functioning of the body (Marshall, 2000). Like caramel (MacKenzie *et al.*, 1992), CP had no adverse effect on the electrolytes of blood.

Urinalysis test was mainly used to evaluate the renal function (Cavanaugh, 1999). In the acute study, the 24-hour urine volumes in the control and treatment groups were similar. However, the total urine volume collected in 0.25% CP-exposed rats of the 6-month chronic treatment had significantly increased ($p<0.05$) (Figure 3.47). With the use of the dip-and-read urine test strips, the levels of several parameters were determined. This is economic, convenient and time-saving, although its aim is to provide a general information on the urine specimen. In this study, the urine specimen did not have any detectable differences. It again implies that the kidneys were functioning properly.

Moreover, urine can provide information on hepatocellular damage. In urine of normal subjects, little urobilinogen is excreted by the kidneys. The urobilinogen is oxidized to urobilin which is a brown pigment. For those having haemolysis or hepatocellular diseases, an increased urobilinogen content will be found in their urine, which will then become deep orange-brown in color (Walmsley and White, 1994).

Thus, the conduction of urinalysis test further proves that a high consumption of

CP in the acute test and a relatively low consumption of CP throughout a long period did not cause any adverse effect to the renal and hepatic systems of the rats.

Macroscopic examination at the end of the acute and chronic toxicity tests did not reveal any lesions in the organs. Further microscopic studies on liver, kidney, thymus and testis showed no morphological changes as compared with the control group. Hence, these organs were not damaged by the application of CP. For caramel, thymi of AC-exposed rats demonstrated a decrease in cortical area, whereas the medullary area appeared to be enlarged (Houben *et al.*, 1992).

In the toxicological studies of CP, although there were some alternations found in the acute and 6-month chronic tests, no treatment-related clinical changes were observed on haematology, blood chemistry and urinalysis tests of the 9-month treatment, although a lower concentration of CP was used when compared to that of the acute test. This indicates that long term exposure to CP did not result in any harmful effects to the major organs such as heart, liver and kidneys. The rats adapted well to CP during the whole experimental period. This also implies that it is safe to humans for long-term consumption.

# Chapter 5 Conclusion

The Chinese chestnut is an economical plant cultivated in different regions of China. The chestnut shell is the non-edible part and is disposed of as agricultural waste. This study reports the preparation of a brown pigment CP from the shells which has a potential use as a natural food colorant.

The CP is water soluble and is very stable. Autoclaving temperature (121°C) did not affect the pigment's absorbance spectrum. The color intensity of CP remained unchanged after a 12-month storage period under normal room lighting condition. These properties allow CP to be used in a wide variety of food items including canned food, bottled beverage, gravy, etc.

Mutagenicity test indicated that CP is not genotoxic. In animal experimentation, results on the haematology, blood chemistry and urinalysis test showed that CP has no acute or chronic toxic effects on the tested rats.

In addition to its coloring property, CP was also shown to have antioxidative activity. A 0.1% CP preparation exhibited antioxidative activity equivalent to 25 mM BHA in the DPPH scavenging assay. This property further expands the pigment's potential application as a natural food preservative (antioxidant).

Further studies on CP include structure analysis of the pigment molecule and development of a more cost-effective procedure for CP preparation.

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